THE EFFECT OF RAUSCHER LEUKAEMIA VIRUS ON HAEMOPOIETIC CELL DIFFERENTIATION IN GENETICALLY DEFECTIVE W/W^{v} AND NORMAL MICE

The work presented in this thesis was supported in part by the Foundation for Medical Research FUNGO, which is subsidized by The Netherlands Organization for the Advancement of Pure Research ZWO, and in part by subsidies of The Netherlands Cancer Foundation 'Koningin Wilhelmina Fonds'.

THE EFFECT OF RAUSCHER LEUKAEMIA VIRUS ON HAEMOPOIETIC CELL DIFFERENTIATION IN GENETICALLY DEFECTIVE W/W^v AND NORMAL MICE

(HET EFFECT VAN HET RAUSCHER LEUKEMIEVIRUS OP HAEMOPOIETISCHE CELDIFFERENTIATIE IN GENETISCH DEFECTIEVE W/W^v EN NORMALE MUIZEN)

proefschrift

ter verkrijging van de graad van doctor in de geneeskunde
aan de Erasmus Universiteit Rotterdam
op gezag van de rector magnificus Prof. Dr. M.W. van Hof
en volgens besluit van het college van dekanen.

De openbare verdediging zal plaatsvinden op
woensdag 28 mei 1986 om 15.45 uur

door

SHOSHANA MERCHAV

geboren te Jerusalem, Israel in 1951

krips repro meppel

PROMOTIECOMMISSIE:

Promotor : Prof. Dr. D.W. van Bekkum

Overige leden: Prof. Dr. R. Benner
Prof. Dr. A.J. van der Eb, Rijksuniversiteit Leiden
Prof. Dr. O. Vos

Co-promotor : Dr. G. Wagemaker

To my husband

CONTENTS

CHAPTER I

INTRODUCTION

1.1 HAEMOPOIETIC CELL DIFFERENTIATION

Haemopoiesis is the process by which mature blood cells, diverse in both structure and function yet limited in lifespan, are continuously replenished throughout life. This process is maintained by pluripotent haemopoietic stem cells (HSC), defined by their capacity to give rise to cells which differentiate into all blood cell lineages, as well as to produce, and thereby maintain, sufficient copies of themselves. Evidence for the existence of a small population of marrow cells which fulfil these criteria, was initially demonstrated in transplantation studies performed in mice. Lethally irradiated recipients reconstituted with marrow cells were found to contain donor-type blood cells of all lineages (Ford et al., 1956; Vos et al., 1956). Eight to ten days after grafting, macroscopically visible nodules containing proliferating haemopoietic cells were detected on the spleens of such recipients (Till and McCulloch, 1961). The functional link between these nodules and the radio-protective capacity of marrow cells was established by the demonstration of the development of a complete haemopoietic system out of the progeny of a single splenic nodule (Trentin and Fahlberg, 1963). The clonal nature of these colonies, some of which contained several haemopoietic cell types, was subsequently demonstrated by studies using chromosomal markers (Becker et al., 1963). Although lymphoid cells could not be visibly identified, they were found to be included among the progeny of spleen colonies (Wu et al., 1968; Nowell et al., 1971), thus establishing the pluripotent nature of the spleen colony forming cells (CFC-S). The self renewal capacity of these cells was further shown by the formation of new spleen colonies after injecting the progeny of single colonies into secondary irradiated recipients (Lewis and Troubaugh, 1964).

Although the pluripotent haemopoietic stem cell corresponding with the CFC-S has been morphologically identified (Van Bekkum, 1977; Van Bekkum et al., 1979), enumeration of spleen colonies in lethally irradiated mice provides the only available assay as yet for the detection and estimation of stem cells within an inoculated cell suspension (Till and McCulloch, 1961). However, the number of cells that form a spleen colony (CFU-S) represents only a fraction of the total population of CFC-S within the inoculum, i.e., those that actually lodge in the spleen. The splenic seeding fraction, f, can be determined by enumeration of the CFU-S recovered from primary recipient spleens at various times following transplantation of the cell suspension, followed by extrapolation of the exponential growth curve to the time of injection (Lahiri et al., 1970). Spleen colony formation has been the endpoint

assay for many studies aimed at gaining fundamental insight into the mechanisms involved in stem cell differentiation as well as self renewal. Recent observations suggest, however, that not all spleen colonies may indeed be derived from pluripotent haemopoietic stem cells (Abramson et al., 1977; Magli et al., 1982; Baines and Visser, 1983). Evidence that spleen colonies of early appearance (i.e., 8 days after transplantation) and transient nature (Magli et al., 1982) seem to originate from differentiating and/or cycling cells, as opposed to the relatively quiescent nature of stem cells which give rise to later-appearing colonies, has recently been provided by stem cell enrichment, coupled with cell cycle-dependent fluorescence intensity staining techniques (Baines and Visser, 1983). Nevertheless, whether these cells meet the functional criteria of stem cells, defined by their capacity to rescue a lethally irradiated host from marrow failure, has yet to be determined.

The concept that the continuous formation of blood cells might be under control of specific humoral regulatory influences received early support from the demonstration that a humoral factor, termed erythropoietin, was involved in the regulation of erythropoiesis (Carnot and Deflandre, 1906; Bonsdorff and Jalavisto, 1948). However, it was not until 1966, when methods for growing colonies of normal haemopoietic cells in semisolid cultures were introduced (Bradley and Metcalf, 1966; Pluznik and Sachs, 1965), that further progress in analysis of haemopoietic cell differentiation and the mechanisms involved in its regulation, could be made.

The first murine haemopoietic colonies to be grown in soft agar were composed of neutrophilic granulocytes and/or macrophages (Bradley and Metcalf, 1966; Ichikawa et al., 1966). The proliferation of cells forming such colonies (termed GM-CFU) was stimulated by using a variety of cell underlayers or media conditioned by various cells (Pluznik and Sachs, 1966; Bradley and Sumner, 1968), mouse or human serum (Robinson et al., 1967; Foster et al., 1968), or even human urine (Metcalf and Stanley, 1967), all of which were subsequently shown to contain specific colony stimulating factors (or CSFs). Although present in a variety of tissues and organs (reviewed by Metcalf, 1981), the physiological significance of these factors and the homeostatic mechanisms which regulate their expression, have not yet been determined.

Following the introduction of techniques for granulocyte-macrophage colony formation, comparable semisolid or viscuous culture systems that could support the proliferation of cells giving rise to erythroid (Stephenson et al., 1971; Axelrad et al., 1974), megakaryocyte (Nakeff et al., 1975; Metcalf et al., 1975), eosinophil (Metcalf et al., 1974), lymphocyte (Metcalf et al., 1975a; Rosenszajn et al., 1975) and basophil/mast cell (Nakahata et al., 1982) colonies, were developed. Wherever examined, colony formation was found to be dependent on the continuous presence of appropriate haemopoietic factors, without which the cells either rapidly died or failed to proliferate (Metcalf and Foster, 1967; Paran and Sachs, 1968; Iscove, 1978; Metcalf and Merchav, 1982).

Extensive investigations into the specific stimulatory requirements as well as the physical and biological properties of marrow cells that give rise to colonies in culture, have shown that these are precursors representing

various stages within a sequential process of differentiation and maturation, maintained under specific regulatory control. A schematic model of haemopoiesis, based on such studies, is given in Figure 1.1. The initial stages involve the differentiation of haemopoietic stem cells into committed or pathway restricted progenitor cells. Stimulation of these progenitors with lineage-specific haemopoietic factors (i.e., erythropoietin, CSF, etc.), induces their proliferation and maturation into functional end cells of the appropriate lineage. It is at this progenitor cell level that the immediate demands for end cells are met.

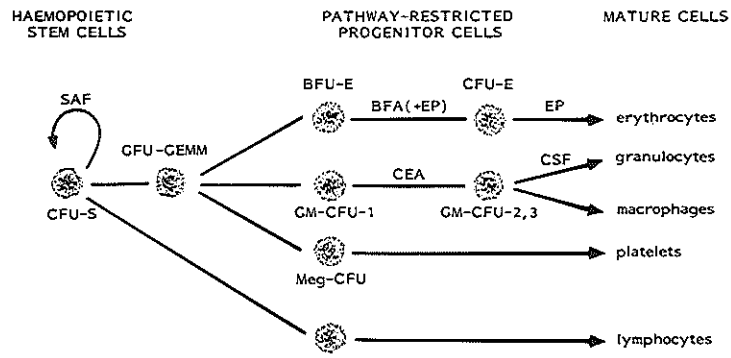


Figure 1.1:
Current scheme of haemopoietic cell differentiation.

Colonies (CFU-GEMM) in which cells of several lineages can be found (Johnson and Metcalf, 1977; Hara and Ogawa, 1978; Johnson, 1980) represent developmentally early precursors in which committment to a single cell lineage has not yet taken place. Similarities in the sedimentation velocities and proliferative states of CFU-S and CFU-GEMM (Hara and Ogawa, 1978; Johnson, 1980), together with evidence of self renewal and spleen colony forming capacities of cells within the latter (Humphries et al., 1979, 1981; Johnson, 1980), suggest that CFU-S and CFU-GEMM represent highly similar cell populations. These colonies appear in cultures supplemented with lectin-stimulated spleen conditioned medium, which serves as a rich source of various haemopoietic factors. The specific stimulatory requirement(s) for mixed colony formation, however, have not yet been analyzed.

Distinct subpopulations of cells representing successive stages of development within a certain cell lineage, have so far been described for the granulocyte-macrophage (Bol et al., 1979; Bol and Williams, 1980) and erythroid (Gregory, 1976; Gregory and Eaves, 1978; Wagemaker, 1980) compartments. As differentiation proceeds, these cells undergo a gradual restriction in proliferative potential and an increase in cell size, density and cycling state (Iscove, 1977; Gregory and Eaves, 1978; Bol and Williams, 1980; Wagemaker, 1980). This process is also accompanied by a gradual acquisition of sensitivity to distinct, lineage-specific haemopoietic factors

(Bol and Williams, 1980; Wagemaker and Peters, 1978; Wagemaker, 1980). Regulators which exclusively stimulate the initial stages of differentiation within each lineage (colony enhancing activity - CEA, and burst forming activity - BFA, respectively), have been identified (Van den Engh and Bol, 1975; Iscove, 1978; Wagemaker, 1978, 1980; Wagemaker and Peters, 1978). Although only partially purified, these early differentiation-inducing glycoprotein molecules seem to share extensive biochemical similarities with each other, as do the physical properties of the developmentally early progenitor cells whose proliferation they stimulate (Van Bekkum et al., 1977; Wagemaker, 1980). The resemblance of the latter to CFC-S (Van Bekkum et al., 1977) further points out to the possible overlap in various detection assays. At present, these are still the main obstacles to the dissection of the early stages of haemopoietic cell differentiation, any further progress in which awaits the use of cell separation and molecular purification techniques of higher resolution.

The mechanism by which stem cell self renewal and differentiation are determined and yet maintained in appropriate equilibrium, is still one of the major unresolved questions in this field. Based on frequency analysis of secondary spleen colony forming cells within primary CFU-S, a "stochastic" model in which the fate of haemopoietic stem cells is instructed by random genetic events, has been proposed (Till et al., 1964; Vogel et al., 1968), and a fixed probability (approx. 0.6) for either to occur, independently determined by several investigators (Vogel et al., 1968; Korn et al., 1973; Metcalf and Moore, 1971; Schofield et al., 1980). Alternatively, a role for external regulatory influences in determining stem cell behaviour has been strongly implicated by in vitro studies resulting in the detection of soluble or humoral regulators which stimulate the proliferation of normally quiescent stem cells. The short term maintenance of CFU-S in culture, coinciding with an initiation of cycling of such cells, was initially found to be stimulated by supernatants of PHA-stimulated mouse spleen cells (Cerny, 1974) as well as by medium conditioned from cultured embryonic fibroblasts (Löwenberg and Dicke, 1976). Cell cycle activation of CFU-S was subsequently found to be induced by bone marrow derived factor(s) (Frindel et al., 1976; Lord et al., 1977), supernatants of lectin stimulated human peripheral blood leukocytes (Wagemaker and Peters, 1978), and serum from endotoxin treated mice (Van Bekkum et al., 1979). The putative molecule exerting this effect, termed stem cell activating factor or SAF (Löwenberg and Dicke, 1976), has been purified from the supernatants of Con-A stimulated mouse spleen cells (Wagemaker, 1980) and characterized as a single 19-20,000 dalton glycoprotein molecule, which could be separated from other known haemopoietic regulator molecules and distinguished on the basis of chemical and/or biological properties from a variety of growth factors and hormones (Wagemaker, 1980; Wagemaker and Merchav, 1981; Wagemaker et al., 1982). It is probably identical to interleukin-3 (Schrader and Clark-Lewis, 1982; Garland and Lewis, 1982; Crompton, 1983; Dorssers and Wagemaker, 1984), which has by now been molecularly cloned and fully characterised (Yokota et al., 1984; Fung et al., 1984) and shown to exert activities designated here as BFA and CEA. SAF has been shown to stimulate the proliferation of stem cells in defined, serum free suspension cultures, resulting in an over 4-fold

increase of CFU-S over a period of 4 days (Wagemaker and Merchav, 1981; Wagemaker et al., 1982). Future studies aimed at evaluating whether SAF only stimulates stem cell self renewal in such cultures, or initiates the differentiation of haemopoietic stem cells as well, may yet provide conclusive evidence for the mechanisms by which haemopoietic stem cell expression is controlled.

1.2 THE W/W^V MOUSE

The first mutation described for the W locus was characterized in homozygous mice by a triad of pleiotropic effects - complete absence of coat pigmentation, sterility in both sexes and severe macrocytic anemia, resulting in perinatal death (de Aberle, 1927). Whereas physiological studies on W gene action were initially hampered by its lethal effect, the finding of a second allele, W^V, with viable homozygotes differing from the original mutants only by a much less severe anemia (Little and Cloudman, 1937), increased the potential for such research. The W/W^V heterozygote, whose anemia is intermediate to that of W and W^V homozygotes, has been the most commonly used mutant for studies on the effect of the W locus on haemopoietic cell differentiation. Although extensively investigated, the nature of the haemopoietic lesions exerted by the W mutation is, however, still poorly understood.

Macrocytic in nature, the anemia of W/Wv mice is primarily due to a reduction in red cell number. The red cell haemoglobin content is proportional to the increased erythrocyte volume and macrocytosis has no effect on red blood cell lifespan (Niece et al., 1963). Elevated levels of circulating erythropoietin (Keighley et al., 1966), correlated with a 50-fold increased level of the enzyme nucleoside deaminase in W/W^V erythrocytes (Harrison et al., 1975), further indicate that under normal atmospheric conditions, erythropoiesis in the W/W^V mouse is characterized by symptoms of increased demand for end cell production.

The anemia of W/W^V mice can be completely and permanently cured by transplantation of haemopoietic cells from +/+ donors (Russell et al., 1956; Bernstein and Russell, 1959). This observation has provided evidence that the appropriate microenvironment for erythropoiesis is not defective in these mutants. Rather, a differentiation defect at the level of the haemopoietic stem cell, has been strongly supported as the major cause of anemia in this mouse.

Evidence for defective stem cell differentiation in mice carrying the W mutation comes mainly from studies involving the spleen colony assay, which is based on the capacity of such cells to give rise to progeny of the erythroid, granulocytic and magakaryocytic lineages within a splenic environment (Till and McCulloch, 1961). Neither spleen nor marrow cell suspensions from W/W^V mice are capable of forming such colonies on the spleen of a lethally irradiated host (McCulloch et al., 1964). Upon subsequent histological examination of such spleens, however, the presence of small numbers of microscopic colonies was revealed (Lewis et al., 1967; Bennett et al., 1968; Van Bekkum and Dicke, 1972). Evidence that these microcolonies represented

self renewing, haemopoietic stem cells, was provided by the formation of similar colonies upon retransplantation of cell suspensions of such spleens into secondary, irradiated recipients (Lewis et al., 1967).

The macroscopic visibility of spleen colonies is mainly due to erythroid progeny (Bleiberg et al., 1965; Curry and Trentin, 1967). However, a decrease in size of all spleen colony types produced by W/W^V stem cells has indicated a restriction in the generation of not only erythroid, but of myeloid precursors as well. Further evidence that erythropoiesis, nevertheless, is more severely affected by the W mutation than myelopoiesis, is demonstrated by the persistent anemia of such mice under steady state conditions, whereas circulating peripheral blood leukocyte as well as platelet numbers do not differ from those found in normal +/+ littermates. The reduced differentiation into these compartments is overcome at subsequent stages of maturation. This is shown, for example, by the maintenance of normal levels of circulating platelets in W/W^V mice, which involves the production of megakaryocytes whose size is unusually large (Ebbe et al., 1973; Ebbe and Phalen, 1978).

Clonal assays in vitro have so far failed to provide information as to the role of the W gene in the process of erythroid differentiation and maturation. Although the frequency of both developmentally early (BFU-E) and relatively mature (CFU-E) erythroid precursors has been found to be reduced in both femur and spleen of W/W^V mice (Gregory and Eaves, 1978; Iscove, 1979; Wagemaker and Visser, 1979), no effect of the W mutation on the rate and extent of proliferation of such progenitors in vitro could be demonstrated; erythroid bursts scored on day 10 of culture did not differ in size from those of normal controls. Whereas these investigators failed to detect any alteration in the sensitivity of erythroid precursors to stimulation with erythropoietin, analysis of the in vitro responsiveness of primitive erythroid precursors to BFA, which stimulates the initial stages of erythroid differentiation, has yet to be performed.

The role of the W gene in the early stages of stem cell differentiation has not yet been investigated, since the early events involved in this process are still largely unknown. Evidence that stem cell responsiveness to external regulatory influences may be affected by the W mutation, is suggested by observations demonstrating that splenic haemopoiesis in this mouse is more severely affected than that of the femur. The frequencies of erythroid and granulocyte-macrophage precursors, as well as megakaryocytes, have been found to be lower in the spleen than in the femur of W/W^V mice, as compared to +/+ littermate controls (Chervenik and Boggs, 1969; Harrison and Russell, 1972; Gregory and Eaves, 1978; Wagemaker and Visser, 1979). The generation of granulocyte-macrophage and erythroid progenitors in lethally irradiated recipients of W/Wv marrow cells, is also more deficient in the spleen than in the femur (Sutherland et al., 1970; Wagemaker and Visser, 1979). Interpretation of these observations in terms of W gene action, however, awaits studies in which the external factors involved in the homeostatic control of stem cell differentiation, have been accurately identified.

As a consequence of the defective capacity of W/W^V stem cells to produce macroscopic spleen colonies, their frequency in this mutant has been

difficult to estimate. Enumeration of histologically detectable microcolonies in recipient spleens of W/W^V marrow grafts has suggested a frequency of approximately 10-25% of normal (Lewis et al., 1967; Van Bekkum and Dicke, 1972). About 10-fold more marrow cells of W/W^V, as compared to +/+ mice, were found by Harrison (1972) to be required for similarly effective radioprotection of lethally irradiated mice.

The lack of a suitable assay for enumeration of haemopoietic stem cells in W/W^V mice has an obvious impact on attempts to perform or to interpret studies in which the effect of the W mutation on haemopoietic cell differentiation are analyzed by comparative methods. It also leaves several central questions as to the role of the W gene in the expression of stem cell properties such as self renewal capacity and homing in the spleen, as yet inaccessible to experimental investigation.

1.3 RAUSCHER LEUKEMIA VIRUS

Since the initial description of experimentally transmissible chicken sarcomas by cell-free tumor extracts (Rous, 1910), the study of RNA tumor viruses has become an major field of intensive research. While the role of retroviruses as a causative agent in human cancer is as yet unresolved, the experimental induction of abnormal growth of animal cells by retroviral infection, has become an indispensable tool for studies of the disruption of normal cellular regulatory mechanisms, resulting in fatal neoplastic disease.

For the experimental haematologist, a small group of murine retroviruses which induce the abnormal proliferation of haemopoietic cells, has enabled studies related to the expression of haemopoietic malignancy, or leukaemia. The rapidity of induction as well as the extreme reproducibility of the ensuing pathological changes, have made retroviral-induced erythroleukemias an exceptionally attractive model for such investigations. The erythroproliferative disease induced by Rauscher Leukaemia Virus (RLV) belongs to this group of diseases.

RLV is a laboratory passaged type C retrovirus, originally isolated by passage of filtrates of leukaemic mouse tissues in newborn BALB/c mice. RLV is actually a complex consisting of a replication defective component (Bentvelzen et al., 1972; Ruta and Kabat, 1980) and a helper virus. The former, termed Rauscher Spleen Focus-Forming Virus (R-SFFV), induces the formation of discrete foci of erythroid cells on the spleens of susceptible mice (Pluznik and Sachs, 1964) and is directly associated with the development of erythroblastosis (Steeves, 1975; Linemeyer et al., 1982). The replication competent helper, Rauscher Murine Leukaemia Virus (R-MuLV), has been found to induce lymphatic or myeloid leukemias in newborn mice after prolonged periods of latency (Reddy et al., 1980; Hagemeijer et al., 1982). In reference to the viral complex, the term RLV is most often used.

The structural relationship between Rauscher MuLV and SFFV components has been demonstrated by nucleic acid hybridization techniques as well as by analysis of proviral encoded proteins (Troxler et al., 1978; Ruta and Kabat, 1980; Bestwick et al., 1983). Such studies have led to the postulation that the SFFV evolved by deletions in the env-gene regions,

which made the SFFV replication-defective, and a recombination at the envelope (env) gene, between the MuLV passaged in the initial leukaemic mouse tissue and endogenous xenotropic virus sequences contained within the host genome, which are capable of propagating only in heterologous species. These events, becoming increasingly apparent as a common mechanism for the evolvement of highly leukemogenic viruses (Lerner, 1978), is schematically shown in Figure 1.2. Although the role of xenotropic envelope gene sequences is not known, their presence in various mammalian tissues as well as their conservation throughout evolution (Levy, 1978), suggests that they may yet be proven to have a significant function in regulatory events related to cellular growth and differentiation.

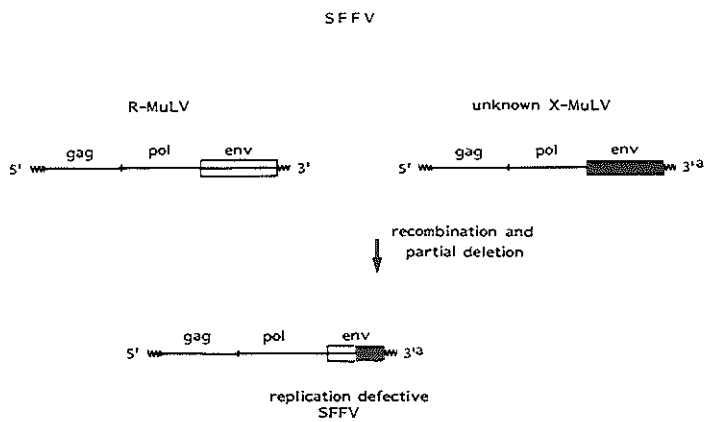


Figure 1.2:
Proposed mechanism for generation of R-SFFV, thought to be a recombinant of R-MuLV and endogenous xenotropic viral sequences (designated X-MuLV) and partial deletions in the env-gene.

Susceptibility to erythroleukemia induction is governed by host genetic factors, the role of which has been established using the Friend (Friend, 1957) strain of erythroleukemia virus (Lilly and Pincus, 1973). Two major loci, FV-1 and FV-2 (termed RV-1 and RV-2, for RLV: Toth et al., 1973), confer susceptibility to the MuLV and SFFV components, respectively. Although not conclusively established, FV-1 probably controls the integration of the MuLV gene into host DNA (Jolicoeur and Baltimore, 1976), whereas the FV-2 locus is assumed to control the expression of SFFV-related sequences (Mak et al., 1979). The development of in vitro culture technology for evaluation of the growth characteristics of murine retroviruses, has enabled the identification of the existence of alleles conferring resistance or susceptibility to these components, among various inbred mouse strains (Lilly and Pincus, 1973).

Infection of susceptible mice with RLV induces a rapidly developing erythroproliferative disease, the most prominent features of which are a continuous proliferation and accumulation of proerythroblast-like cells in the

spleen and liver, bone marrow and finally, peripheral blood. Apart from this major manifestation, Rauscher disease is also characterized by leukocytosis, thrombocytopenia and anemia, all of which are detectable within 3-5 weeks after viral infection. Lymphoid or myeloid leukemias have been observed in mice surviving the initial erythroblastosis (Rauscher, 1962; Boiron et al., 1965; Pluznik et al., 1966), or in newborn C57BL mice or rats, which do not manifest the initial phase at all (Rauscher, 1962; Boiron et al., 1965). Whereas the appearance of leukaemia in C57BL mice or rats can be conclusively attributed to the helper component within the viral complex, the role of the SFFV component in induction of such leukemias as a subsequent stage to erythroblastosis, is difficult to establish.

The nature of Rauscher disease and its various manifestations has given rise to much controversy, as yet unresolved, regarding the neoplastic nature of Rauscher disease, as well as the primary viral target. The confusion and disagreement in relation to the first issue is most vividly exemplified by the various connotations used in reference to this disease, i.e., erythroblastosis, erythroleukemia, erythrocytosis, or erythroid hyperplasia. Arguments against as well as in favor of the neoplastic nature of Rauscher disease, have been presented. Dunn and Green (1966) claimed that the rapid reaction to viral infection, as well as the detection of various stages of erythroid maturation, indicating that differentiation was still occurring, are not characteristic features of a leukaemic process. The validity of the latter argument, however, is rather doubtful. Chronic myeloid leukaemia is a typical example in which normal differentiation of myeloid cells is clearly apparent.

The massive accumulation of proerythroblasts in the spleen (60-80% of all nucleated cells, de Both et al., 1978) greatly exceeds the compensatory needs due to anemia in RLV-infected mice. This observation has been variably interpreted, as well. Favouring a malignant process, a maturation arrest of erythroid precursors, induced by erythropoietin- dependent expression of the proviral genome, has been proposed (Brommer, 1972; Brommer and Bentvelzen, 1973). Conversely, the persistent anemia has been claimed to result from extensive proerythroblast-like cell death in the spleen, with immunological cytotoxicity and premature enucleation contributing to the destruction of these cells (de Both et al., 1978). The fact that the majority of the proerythroblast-like cells does not form erythroid colonies in vitro (Hasthorpe and Bol, 1979), does not, however, distinguish between both these alternatives.

Whereas continued responsiveness to the humoral regulator erythropoietin has been demonstrated both in vivo (Dunn et al., 1966; Brommer, 1972) and in vitro (Opitz et al., 1977; Hasthorpe, 1978; Gallicchio and Murphy, 1981), evidence suggesting autonomous, or BFA-independent BFU-E growth, from femurs and/or spleens of RLV-infected mice, has been presented (Walma and Wagemaker, 1979; Ostertag and Pragnell, 1981).

Initial studies of subcutaneous transplantation of RLV-infected tissues resulted in a repetition of the erythroblastic reaction in haemopoietic organs by recipient cells, with growth at the site of transplantation absent (Brommer, 1972; Brommer and Bentvelzen, 1973). Nevertheless, recent advances in tumour and culture technology, have resulted in the isolation of a variety of transformed erythroid, lymphoid and myeloid cell lines from

similarly transplanted livers and spleens of RLV- infected mice (de Both et al., 1978a; Hagemeijer, 1982).

Comparable to the role of normal cellular sequences, or "onc" genes, in expression of the transforming capacity of avian and mammalian retroviruses (reviewed by Duesberg, 1979), the recombinant envelope sequences of the SFFV component have been considered to be the putative "leuk" gene of Rauscher virus (Troxler et al., 1980; Scolnick, 1982). Since the neoplastic nature of Rauscher erythroblastosis has not, however, been conclusively established, this assignation is still premature. Final resolution of this question awaits the initiation of studies in which the in vivo consequences following transfection of target haemopoietic cells with the SFFV component, can be evaluated. With this goal in mind, the major controversial issue regarding the viral target, must first be resolved.

With the continuous proliferation and accumulation of proerythroblast-like cells being the most prominent manifestation of RLV infection, it is not surprising that major attempts were initially focussed on finding a viral target among cells of the erythroid compartment. Pluznik et al. (1966) demonstrated that spleen focus formation following RLV infection, varied directly with perturbations leading to a rise or decline in erythropoietin levels. Suppression of erythropoietin levels was found to significantly delay the splenic enlargement (Dunn et al., 1966; Brommer, 1972) and also resulted in considerable prolongation of survival time (Dunn et al., 1966; Weitz-Hamburger et al., 1973). Rauscher disease was thus initially considered to be an erythropoietin- dependent erythroleukemia, implying erythroid precursors to be the main viral targets. Subsequent investigations, however, provided evidence at variance with this concept, suggesting the haemopoietic stem cell to be the primary target of Rauscher virus.

Seidel (1972) suggested that the initial tissue response to RLV infection, characterized by a neutropenia, thrombopenia and reticulopenia, was due to the involvement of a common stem cell. Strong arguments for his concept of the primary involvement of the stem cell in Rauscher disease, were provided by subsequent studies (Seidel, 1973), demonstrating that the increase in splenic CFU-S, occurring before the development of any pathogenic manifestations, could not be inhibited by suppressing erythropoietin levels. Seidel further observed that whereas bleeding or stimulation of mice with endotoxin prior to RLV infection greatly enhanced the extent of subsequent splenomegaly, pretreatment with Myleran had a suppressive effect. Although he explained the outcome of these latter manipulations to alterations of CFU-S numbers in the spleen, such experimentation, however, does not rule out direct alterations within the erythroid compartment, as well.

A detailed investigation into the effect of RLV infection on various haemopoietic compartments, employing clonal assays in vitro, has revealed an early exponential rise in the spleen, of not only erythropoietin dependent precursors, but of precursors of granulocytes and macrophages, as well as megakaryocytes (Iturriza and Seidel, 1974; OKunewick and Chervenik, 1977; Hasthorpe, 1978). Although a common viral target, the haemopoietic stem cell, may again be implied by these observations, this phenomenon may also be due to a direct effect of the virus on individual cell types (OKunewick, 1977).

In defining a viral target, an event related to transformation, is normally assumed. In spite of the strong evidence for the primary involvement of the haemopoietic stem cell in the expression of Rauscher disease, direct evidence for altered stem cell expression, however, has not been presented. The presence of viral antigens on stem cells from RLV-infected mice (Brommer, 1972; Brommer and Bentvelzen, 1974) indicates no more than proviral integration. Both the spleen colony types produced by CFU-S as well as their 30-day radioprotective capacity, have not been found to be altered by RLV infection (Brommer, 1972; Brommer and Bentvelzen, 1974). Thus, before assessing the mechanism(s) by which the putative "leuk" gene exerts its effects on the haemopoietic system, the controversial issue relating to the viral target, has yet to be resolved.

1.4 OUTLINE OF THE STUDY

The main objective of the studies presented in this thesis is to clarify the controversial issue concerning the primary role of the stem cell in Rauscher erythroblastosis. Genetically anemic mice carrying the W mutation, characterized by a stem cell differentiation defect, have been chosen as an experimental model for these investigations.

In Chapter 3, the susceptibility of W/W^V genotypes to Rauscher infection is examined by several known parameters, such as spleen weight, peripheral blood values, etc. The relationship between the development of Rauscher disease in these mutants and a restoration of their stem cell differentiation defect, is evaluated by the spleen colony assay.

Chapter 4 deals with studies of the functional properties of RLV-infected W/W^V stem cells, assayed in lethally irradiated W/W^V recipients. The 30-day radioprotective capacity of RLV-infected W/W^V CFU-S, the numerical and physical properties of erythrocytes generated by these cells, as well as the capacity of RLV-infected W/W^V stem cells to give rise to tissue mast cells in W/W^V recipients, are investigated.

Chapter 5 describes the development and establishment of serum free cultures for the stimulation of stem cells and progenitor cells with specific regulators in vitro. The elimination of serum, known to contain specific haemopoietic regulators, is a prerequisite for accurate investigations into the role of regulatory mechanisms in the expression of haemopoietic disorders.

In an attempt to elucidate the mechanism(s) by which RLV infection exerts its effects on the haemopoietic system, with special relevance to the early stages of haemopoietic cell differentiation, the effect of RLV infection on the sensitivity of stem cells and progenitor cells to specific regulators in vitro, is examined in both normal and genetically anemic W/W^V mice. These investigations are described in Chapter 6.

Chapter 7 describes studies performed in lethally irradiated recipients, in which the effect of the W mutation and RLV infection on stem cell differentiation and self renewal in both the spleen and femur, are investigated.

In the final chapter, the information obtained in these studies is incorporated into a model relating the primary involvement of the haemopoietic stem cell in the development of Rauscher erythroblastosis.

CHAPTER II

MATERIALS AND METHODS

2.1 EXPERIMENTAL ANIMALS

All mice were bred and maintained under specific pathogen free conditions until used.

2.1.1 W/W^V mice

Male and female mutant W/W^V mice of C57BL/Ka, DBA/2 and (C57BL/Ka x DBA/2)F1 or BD_2F1 strains, were used for these studies. These mice, obtained by crossing W/+ and $W^V/+$ heterozygotes, were bred at the animal colony in Rijswijk. DBA/2 mice carrying the W or W^V allele were descendants of C57BL/Ka heterozygotes, obtained by outcrossing and at least 12 subsequent backcrossings onto a DBA/2 background.

BD_2-W/W^V littermates (BD_2-W/+, $W^V/+$, +/+) were used in several experiments as controls or as lethally irradiated recipients.

2.1.2 Irradiation of mice

Recipient mice used for spleen colony assays and survival studies were 12-16 weeks of age. They were subjected to lethal total body irradiation by gamma rays from a ^{137}Cs source, delivered at a dose rate of 0.87 $Gy.min^{-1}$. Irradiated controls which had not been transplanted with haemopoietic cells died within 12 to 15 days.

2.2 RAUSCHER VIRUS (RLV)

Rauscher virus was obtained from a stock originally received in 1977 by the Radiobiological Institute TNO from the Netherlands Cancer Institute, Amsterdam, to which it was provided in 1967 by Dr. F. Rauscher Jr., National Cancer Institute, Bethesda, Maryland.

2.2.1 Preparation of cell free extract and administration of virus

Enlarged spleens (more than 2 grams) of BALB/c mice infected three weeks previously with RLV, were removed and homogenized in an Omnimixer (Sorvall, type OM 1220) with a 5-fold volume per unit weight ($ml.gr^{-1}$) of ice-cold phosphate buffered saline (PBS), for a full minute at full speed (19×10^3 rpm). The resulting homogenate was spun for 30 minutes at 20×10^3

rpm at 4°C in a Beckman (Model J-21B) centrifuge. The supernatant containing the virus stock was aliquoted in volumes of 2-4 ml into plastic vials, sealed and stored in liquid nitrogen until use. Immediately after thawing, the cell free extract was administered intraperitoneally into 4-6 week-old recipient mice. An ampule which had been thawn once was never refrozen for future use.

2.2.2 Spleen focus forming assay

The amount of R-SFFV contained within the cell free extract was determined by the spleen focus forming assay, performed according to the method described by Pluznik and Sachs (1964). Groups of 8 female DBA/2 mice aged 10 weeks were injected intravenously with 0.5 ml of 10-fold serial dilutions of cell free viral extract. Eight days later these mice were killed and their spleens fixed in Telleyesnickzy's solution (2% formalin, 5% acetic acid, in 70% ethanol). Spleen foci were enumerated 1 hour after fixation.

2.3 PREPARATION OF CELL SUSPENSIONS

Cell suspensions were prepared in Hanks' Balanced Salts Solution (HBSS) buffered at pH 7.2 with 10 mM Hepes Buffer (Merck), the final osmolarity adjusted to 300 mOsm. Mice were killed by cervical dislocation under aether anesthesia. Spleens and femurs were removed and placed in icecold HBSS in separate 50 mm Petri dishes. Cells were flushed from the femoral cavity using a syringe adapted with a 25 gauge needle. Single cell suspensions were prepared by repeated gentle pipetting.

Spleen cell suspensions were prepared by first cutting the spleens into fine pieces with scissors, followed by gentle teasing through a nylon sieve. Preparation of single cell suspensions of enlarged spleens of RLV-infected mice was facilitated by the addition of approximately 50 µg DNAse per spleen (82,000 Dornase Units/mg Calbiochem II), prior to pipetting. All cell suspensions were washed (400 g, 10 min) and resuspended in fresh medium prior to counting. Nucleated cell counts were performed in a haemocytometer using Türk's solution (0.005% Crystal Violet, 1% acetic acid, in H_2O).

2.4 SPLEEN COLONY ASSAY

The spleen colony assay was performed according to Till and McCulloch (1961). Appropriate numbers of nucleated cells, estimated so as to give a range of 10-20 colonies per spleen, were suspended in HBSS and injected in 0.5 milliliter volumes into the tail vein of lethally irradiated syngeneic or congenic recipient mice. Eight to ten mice were used per experimental group. Irradiated mice which had not been transplanted with haemopoietic cells, served as controls for endogenous colony formation. After 10 days, the mice were killed, their spleens removed and fixed in Tellyesniczky's solution. Macroscopically visible nodules were scored as spleen colony forming units (CFU-S) several hours later.

2.4.1 Secondary spleen colony formation

Assessment of stem cell self renewal capacity was performed by enumeration of CFU-S found within primary spleen colonies, in the following manner: a group of 13 lethally irradiated recipients was transplanted with a given haemopoietic cell suspension and killed 10 days thereafter. Eight spleens were removed and fixed for spleen colony enumeration. The remaining (5) spleens were pooled and resuspended in HBSS. Dilutions of these cell suspensions were transplanted into 8 lethally irradiated (secondary) recipients. There were killed 10 days later, their spleens fixed and used for enumeration of secondary colonies. These served for evaluation of the number of secondary CFU-S per primary CFU-S.

2.4.2 Determination of the seeding efficiency (f-factor)

The f-factor indicates the fraction of stem cells within an injected haemopoietic cell suspension, which lodge in the spleen and give rise to macroscopic spleen colonies (Siminovitch et al., 1963). Lahiri et al. (1969, 1970) showed that the number of CFU-S in the spleen sharply decreases during the first 24 hours and provided evidence that f has to be derived from extrapolation of the exponential growth curve to the time of injection. Enumeration of CFU-S recovered 2 hours post grafting in spleens of recipients which had been irradiated 3 days previously was further shown to give the same value for f as obtained by extrapolation.

Determination of the seeding efficiency was performed in a manner similar to that described for secondary spleen colony formation (2.4.1) except that the spleen donors for evaluation of secondary spleen colony numbers were mice which had been irradiated 3 days previously (n = 5) and killed 2 hours after transplantation of the given cell suspension. The f-factor was calculated as the ratio between the number of CFU-S obtained 2 hours after transplantation and the total number of spleen colonies within the injected cell suspension determined in separate group (n = 8) of mice.

2.5 CLONAL ASSAYS IN VITRO

2.5.1 Methylcellulose cultures

The method employed for viscuous cultures of haemopoietic progenitors is a modification of the one previously described (Wagemaker and Visser, 1980), which was based on methods described by Iscove, Till and McCulloch (1972) and Guilbert and Iscove (1976). The basic medium used was a modification of Dulbecco's medium (Dulbecco's MEM, Gibco 430-1600), supplemented with several amino acids, vitamin B12, biotin and Na-pyruvate (as fully described by Wagemaker and Visser, 1980), containing 4.5 $g.l^{-1}$ glucose, 3.7 $g.l^{-1}$ $NaHCO_3$, 10^5 units.l^{-1} penicillin and 100 $mg.l^{-1}$ streptomycin sulfate, at 300 mOsm. This medium was further supplemented with 5% foetal calf serum (Flow), 1% (w/v) delipidated, deionized (Worton et al., 1969; Iscove et al., 1980) bovine serum albumin (BSA, Sigma), 4×10^{-6} M human transferrin, saturated with $FeCl_3$, 10^{-7} M $Na_2SeO_3.5H_2O$ (Merck), 20 µg/ml egg lecithin (Merck), 10^{-4} M 2-mercaptoethanol (Merck) and 10^{-3} $g.l^{-1}$ nucleo-

sides (adenosine, guanine, uridine, cytidine, 2'deoxyadenosine, 2'deoxyguanosine, 2'deoxycytidine, thymidine), using 0.8% (w/v) methylcellulose (Methocel A4M Premium grade, Dow Chemical Co.) as a viscuous agent. All reagents were of the purest grade available.

One and a half milliliter cultures containing bone marrow or spleen cells and the appropriate haemopoietic factor (see 2.5.2), were set up in 5 ml plastic tubes (Falcon, 2058). After rapid shaking of the contents, one milliliter aliquots were removed with a syringe adapted with an 18G needle and plated in 35 mm (Falcon, 1008) petri dishes. Cultures were incubated at 37°C in an atmosphere of 7.5% CO_2 in air and 100% humidity until scored. All progenitor cells (BFU-E, CFU-E, GM-CFU) were assayed in the same serum supplemented culture system.

Serum free cultures for detection of these progenitors were the same as described above, except that lecithin was replaced by linoleic acid (Merck) and cholesterol (Sigma), both at a final concentration of 1.5×10^{-5} M. These were dissolved in 96% ethanol at an appropriate concentration (7.5×10^{-3} M), so as not to exceed a final volume of 0.2% ethanol in culture. Serum-free cultures for stimulation of GM-CFU with GM-CSF were further supplemented with 10^{-6} M hydrocortisone Sodium Succinate (Sigma). BFU-e and CFU-e were assayed in serum-free cultures supplemented with 0.2 mM hemin (Protoporphyrin IX, Sigma).

2.5.2 Scoring of colonies

Colonies were scored using an inverted microscope (Zeiss).

CFU-E were scored in cultures stimulated by EP at a super-saturating concentration of 0.5 unit.ml^{-1}. The clusters were scored after 2-3 days according to the criteria established by Iscove, Sieber and Winterhalter (1974), at a final magnification of x 200.

BFU-E were assessed in cultures stimulated by a BFA and EP at a saturating concentration of 1 unit.ml^{-1}. Bursts were scores after 9-10 days on the basis of their characteristic clustered appearance (Axelrad et al., 1974) and red colour (Wagemaker, 1978), at a final magnification of x 79.

GM-CFU were assayed in cultures stimulated by a saturating concentration of M-CSF or GM-CSF. These were scored after 7 days at a final magnification of x 30.

2.6 SUSPENSION CULTURES FOR STEM CELL PROLIFERATION IN VITRO

The method for assaying the proliferation of haemopoietic stem cells stimulated by SAF in serum free suspension cultures is a modification of the one described by Wagemaker and Peters (1978), which is based on the method initially described by Löwenberg and Dicke (1976).

Bone marrow cells (3×10^5 or 10^6 cells) were cultured in plastic round bottomed tubes (Falcon nr. 2057) containing 1 ml of modified Dulbecco's medium, supplemented with 0.25% (w/v) BSA and the same concentrations of transferrin, Na_2SeO_3, nucleosides, linoleic acid, cholesterol and mercaptoethanol, as described in 2.5.1. These cultures were further supplemented

with 10^{-6} M Hydrocortisone Sodium Succinate (Sigma) and 10^{-6} M Isoproterenol (Sigma). Cultures were stimulated with a saturating dose of SAF purified from medium conditioned by Concanavalin A stimulated mouse spleen cells (2.7.5).

Loosely capped tubes were placed in an incubator at 37°C, 7.5% CO_2 and 100% humidity in air for a period of 4 days. Cultures were terminated by adding a 5- to 10-fold volume of ice cold HBSS. The diluted suspensions were injected intravenously in 0.5 ml volumes into 8-10 lethally irradiated syngeneic recipients. These were killed 8-10 days later, their spleens removed and fixed for enumeration of macroscopically visible colonies.

The extent of stem cell enrichment was calculated as the ratio between the final yield of CFU-S after 4 days in culture and the initial number of CFU-S in culture, determined by injecting dilutions of the same cell suspension into 2 groups (n = 8) of lethally irradiated mice. These received 3 x 10^4 or 6 x 10^4 marrow cells per mouse.

2.7 PREPARATION OF STIMULI

2.7.1 Preparation of conditioned medium from Con-A stimulated mouse spleen cells (MSCM)

Medium conditioned by lectin stimulated mouse spleen cells is a rich source of a variety of haemopoietic regulators (Metcalf and Johnson, 1978). Culture conditions for preparation of MSCM are the same as described for serum-supplemented viscuous cultures (2.5.1) except that methylcellulose is omitted, the fetal calf serum concentration is 2% and the transferrin concentration reduced 16-fold (i.e. 2.5 x 10^{-17} M). Spleen cells of C57BL/Ka female mice, at a concentration of $10^6.ml^{-1}$, were stimulated with Con A (Pharmacia) at 1 $\mu g.ml^{-1}$. Cultures were incubated in flasks (Corning) for 7 days. The supernatant was harvested after centrifugation at 500 g (10 min, room temperature) and used for purification of GM-CSF, BFA and SAF, as described in the following sections.

2.7.2 Granulocyte-macrophage coloning stimulating factor (GM-CSF)

GM-CSF was purified from the supernatant of Concanavalin-A stimulated mouse spleen cells, using a sequence of affinity chromatography (ConA-Sepharose), gel filtration (Sephacryl S200), ion exchange chromatography (DEAE-Sepharose, pH 8.0) and gel filtration (Sephadex G75). The resulting preparation was analysed by polyacrylamide gel electrophoresis and chromatofocussing using fast protein liquid chromatography (FPLC), which demonstrated that the biological activity was associated to a single protein with a molecular weight of 29,000 dalton, which eluted at a pH of 4.1. The preparation did not contain detectable levels of factors stimulating BFU-E or CFU-S using appropriate assays (2.5.1, 2.6). It stimulated colonies composed of macrophages and granulocytes at concentrations that approached saturation at 3.5 $\mu g.ml^{-1}$. The major contaminant of the preparation was bovine serum albumin, used to prepare the original spleen conditioned medium.

2.7.3 Macrophage colony stimulating factor (M-CSF)

Pregnant mouse uterus extract (PMUE) was prepared as described by Bradley et al. (1971) and Stanley et al. (1972). M-CSF was purified from PMUE using a sequence of affinity chromatography (ConA-Sepharose), ion exchange chromatography (DEAE-Sepharose, pH 8.0), hydrophobic interaction chromatography (phenyl-Sepharose) and repeated polyacrylamide gel electrophoresis. The resulting preparation gave a single 61,000 dalton molecular weight band upon SDS polyacrylamide gel electrophoresis. It eluded as a single peak at a pH of 4.8 when subjected to chromatofocussing using FPLC. The preparation stimulated exclusively macrophage colonies at any concentration tested. For routine purposes, a semipurified preparation of M-CSF, subjected only to affinity and ion exchange chromatography, was used.

2.7.4 Erythropoietin

Erythropoietin was isolated from sera of mice which had undergone lethal irradiation, followed by administration of phenylhydrazine chloride (Merck), 2 mg/mouse, 2 and 4 days thereafter. The supernatant of a 40% $(NH_4)_2SO_4$ precipitate was subjected twice to ion exchange chromatography (DEAE-Sepharose, pH 8.0) and eluted with 0.25 M and 0.1 M NaCl, respectively. Activity of the fractions was assayed using 14-day old fetal liver cells from C57BL/Rij mice, calibrated against the International Reference for Erythropoietin as control.

2.7.5 Burst Forming Activity (BFA), Colony Enhancing Activity and Stem Cell Activating Factor (SAF)

Spleen conditioned medium was prepared and described by Wagemaker and Visser (1980). An about 1600-fold increase in specific activity was achieved by a simple two step procedure, consisting of adsorption to Con A-Sepharose (Iscove, 1978) and gel filtration using Sephadex G150 as described (Wagemaker, 1980). The resulting concentrate contains SAF, BFA, CEA and a low concentration of GM colony stimulating activity; it was used routinely as a source of BFA and CEA. Starting from this concentrate, SAF was partially separated from BFA and enriched to an over-all increase in specific activity of 50,000-fold by adsorption to DEAE-Sepharose, equilibrated with 0.01 M Tris-HCl, pH 8.0 and eluted with a gradient of 0-0.5 M NaCl in the same buffer. Under these conditions, SAF elutes at 0.02-0.03 M NaCl and is separated from 98% of the contaminating proteins. The resulting material was termed stage 3 SAF and used throughout the experiments.

2.8 PERIPHERAL BLOOD ANALYSES

2.8.1 General

Mice were lightly anaesthesized with aether and bled by orbital puncture into plastic vials containing a small amount of EDTA. Blood smears were air dried and stained with May-Grünwald-Giemsa. Hematocrits were read after

centrifugation in a microhaematocrit centrifuge (International model B). Reticulocytes were scored after staining with Brilliant Cresyl blue. All other blood cells (leukocytes, erythrocytes, thrombocytes) were counted using the Elzone Particle Channelyzer (Particle Data, Inc., Cheltenham, England). Mean red cell or corpuscular volume (MCV), was calculated as the ratio between hematocrit and red blood cells.

2.8.2 Red blood cell size profile

Analysis of the red cell size profile was performed using the Elzone Particle Channelyzer, attached to a 256-channel pulse height analyzer. The channels were calibrated for cell volume by running particles of 4.88 µm diameter through a 76 µm orifice at a setting of: Threshold - 4%, Current -4.5, Gain - 5. The calibrated volume increment per channel was 0.871 fl.

For analysis of red cell size distribution, 40 microliters of blood were diluted 62,500 fold with Isoton (Coulter Electronics, W. Germany). Suspensions of 0.25 ml were processed for red cell size. For comparative purposes, equal numbers of cells of various samples were analyzed. The time set for running each sample was calculated according to the red blood cell concentration within that sample.

2.9 HISTOLOGY

For histological examinations, the organs were fixed in Tellyesniczki's solution or in 4% buffered formalin. These were processed for histology at the Pathology Department of the Institute for Experimental Gerontology at the TNO. Sections were routinely stained with haematoxylin-phloxin-saffran. Sections examined for the presence of tissue mast cells (i.e., skin, stomach) were stained metachromatically with toluidine blue, at a pH of 2.0.

2.10 STATISTICAL ANALYSIS

Standard deviations were calculated on the assumption that colony counts are Poisson distributed (Blackett, 1974). Statistical analyses were performed using a two-tailed Students' t-test.

CHAPTER III

REPAIR OF MACROSCOPIC SPLEEN COLONY FORMING CAPACITY OF W/W^V STEM CELLS BY RAUSCHER LEUKAEMIA VIRUS

3.1 SUSCEPTIBILITY OF W/W^V MICE TO RAUSCHER LEUKEMIA VIRUS

The use of the W/W^V mouse as an experimental model for investigation of the primary involvement of the haemopoietic stem cell in the development of Rauscher erythroblastosis, required initial assessment of the susceptibility of this mutant to RLV infection. For this purpose, several strains of mice carrying the W/W^V alleles were bred. The peripheral blood values of these mutants are given in Table 3.1. All three strains of W/W^V mice have similar peripheral red blood cell values, consistent with those representing moderate macrocytic anemia, as previously described (Russell and Bernstein, 1966). Although genetically favorable for susceptibility to R-SFFV, DBA/2-W/W^V mice exhibited extremely poor viability, with only one half of the offspring (14% of all littermates) surviving after weaning. Hybrid vigor was clearly apparent in BD_2F1-W/W^V mutants, both by frequency at birth (28%) and survival thereafter (94%).

Table 3.1

PERIPHERAL BLOOD VALUES OF W/W^V MICE

	DBA/2-W/W^V (n = 3)	C57BL/Ka-W/W^V (n = 5)	BD_2F1-W/W^V (n = 9)	+/+* (n = 5)
haematocrit (%)	26 ± 6.5	31 ± 1.8	30 ± 6.8	42 ± 1.8
erythrocytes ($x10^9.ml^{-1}$)	4.0 ± 1.1	4.7 ± 0.4	4.4 ± 1.3	9.1 ± 0.2
mean corpuscular volume (MCV) (fl)	69 ± 8.1	65 ± 5.2	60 ± 5.5	45 ± 1.5
haemoglobin (mM)	7.5	7.0	7.5	14.0
reticulocytes (%)	12.4 ± 8.7	7.5 ± 2.9	7.1 ± 2.4	3.5 ± 0.6
leukocytes ($x10^6.ml^{-1}$)	12.5 ± 7.5	4.5 ± 0.9	4.3 ± 2.6	3.2 ± 1.9
platelets ($x10^6.ml^{-1}$)	4.4 ± 0.3	3.2 ± 0.2	5.6 ± 0.6	5.3 ± 0.7

*BD_2 +/+ littermates of similar age.
Values represent mean ± SD of individual determinations.

3.1.1 Spleen weight and peripheral blood analysis

Using splenic enlargement as a measure for susceptibility to Rauscher virus, the spleens of W/W^V mice of DBA/2, C57BL/6 and BD_2F1 strains were weighed 21 days after viral administration. Parental +/+ C57BL/6, DBA/2 and BD_2 +/+ littermates of similar age were used as controls. The results are shown in Table 3.2. Both DBA/2 and BD_2-W/W^V mice showed an approximately 10-fold increase in splenic weight, as compared to uninfected controls. The splenic enlargement in +/+ controls of these two strains was significantly greater ($p < 0.01$), an observation which has also been reported in similar studies performed with Friend virus (MacDonald et al., 1980).

W/W^V and +/+ genotypes of C57BL/6 mice were resistant to RLV-induced splenomegaly. This is in accordance with the $Fv\text{-}2^r$ genotype of this mouse strain, which confers resistance to the SFFV component of the viral complex (Lilly and Pincus, 1973).

W/W^V mouse strains found to develop enlarged spleens after infection with Rauscher virus, were bled for peripheral blood evaluation. Figure 3.1 demonstrates the presence of aberrant distorted erythrocytes, "smudge" cells and circulating nucleated red cells, typically found at the progressive stages of Rauscher disease (Rauscher, 1962, Brommer, 1972; OKunewick et al., 1972).

Peripheral blood values of RLV-infected W/W^V mice are shown in Table 3.3. They are also characteristic for RLV infection, demonstrating leukocytosis, reticulocytosis, anemia and slight thrombocytopenia.

Table 3.2

SPLENIC WEIGHT OF RLV-INFECTED W/W^V AND +/+ CONTROL MICE
(3 weeks post-infection)*

strain	spleen weight (g) ± SD	FV genotype FV-1	FV-2
DBA/2 +/+	2.0 ± 0.3	nn	ss
DBA/2 W/W^V	1.23 ± 0.6		
C57BL +/+	0.16 ± 0.02	bb	rr
C57BL W/W^V	0.21 ± 0.04		
BD_2F1 +/+	1.8 ± 0.8	nb	rs
BD_2F1 W/W^V	1.1 ± 0.5		
controls**	0.11 ± 0.01		

* 5-6 week old mice were injected intraperitoneally with 0.4 ml cell-free extract containing $16.4 \pm 1.8 \times 10^4$ $SFFU.ml^{-1}$, determined as described in Chapter 2.2.2.
**uninfected +/+ BD_2 mice of similar age.

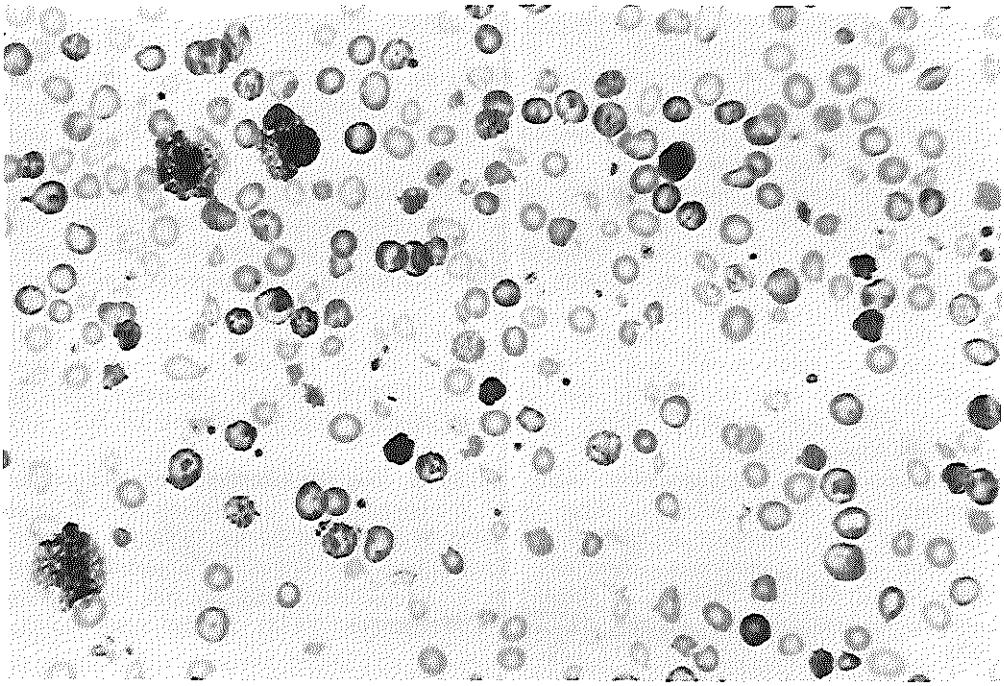


Figure 3.1:
Peripheral blood morphology of RLV-infected W/W^V mice, 3 weeks post-RLV infection.

Table 3.3

PERIPHERAL BLOOD VALUES OF RLV-INFECTED AND CONTROL BD_2-W/W^V MICE
(3 week post-infection)

	W/W^V-RLV (n = 6)	W/W^V (n = 9)
haematocrit	20.0 ± 4.0	30.0 ± 6.8
erythrocytes ($x10^9.ml^{-1}$)	3.3 ± 1.2	4.4 ± 1.3
MCV (fl)	57.0 ± 1.9	60.0 ± 5.5
platelets ($x\ 10^6.ml^{-1}$)	3.2 ± 1.2	5.6 ± 0.6
reticulocytes (%)	>10	7.1 ± 2.4
total nucleated cells ($x10^6.ml^{-1}$)	43.0 ±39	4.3 ± 2.6
normoblasts (%)	28.0 ± 6	5.0 ± 2
leukocytes ($x10^6.ml^{-1}$)	31.0 ± 8	4.1 ± 0.23
- granulocytes (%)	36.0 ± 8	49.0 ±13
- lymphocytes (%)	64.0 ± 9	53.0 ±13
- monocytes (%)	-	-

Values represent mean ± SD of individual enumerations.

3.1.2 Haemopoietic progenitors in the spleen and femur

Infection of susceptible mice with Rauscher virus results in an exponential rise in developmentally early (BFU-e) and mature (CFU-e) erythroid precursors, as well as progenitors of granulocytes and macrophages (GM-CFU) in the spleen (OKunewick, 1973; Opitz et al., 1977; Seidel and Opitz, 1978; Hasthorpe, 1978).

These events were studied in RLV-infected BD_2-W/W^V mice, using infected BD_2 +/+ littermates of similar age and sex, for comparison. The data, presented as the ratio between RLV-infected and control (uninfected) values in the spleen (i.e. splenic weight, cellularity, progenitor cells) are demonstrated in Figure 3.2.

Of all progenitors assayed, the increase in splenic CFU-E is most prominent in both W/W^V and +/+ genotypes, being 100-300-fold higher than controls. The rise in splenic GM-CFU and BFU-E closely parallels that of splenic cellularity in W/W^V mice, whereas GM-CFU concentrations in BD_2 +/+ mice are clearly higher.

Haemopoietic progenitors were assayed in the femurs of Rauscher infected BD_2 mice, as well. The data are presented in Figure 3.3. Total nucleated cells, as well as CFU-E and BFU-E appeared to be reduced in both +/+ and W/W^V genotypes, whereas fluctuations of GM-CFU around normal were found for the former. A depletion of progenitor cells in the femur has been demonstrated by others as well (Hasthorpe, 1978; Seidel and Opitz, 1978).

3.1.3. Mortality following Rauscher infection

Infection of susceptible mice with high doses of Rauscher virus results in rapidly developing erythroblastosis, with death of the recipients ensuing within 3-5 weeks (Rauscher, 1962; Brommer, 1972; O'Kunewick et al., 1972).

The survival of BD_2-W/W^V mice following RLV administration was examined and compared to that of BD_2 +/+ littermates, as well as to the parental DBA/2 strain. BALB/c mice, whose mortality pattern after RLV infection has already been documented (Brommer, 1972), served as controls. The survival curves are presented in Figure 3.4.

A specific pattern of mortality in all three mouse strains, as a result of Rauscher infection, is clearly evident. Death following RLV infection was rapid in BALB/c mice, as previously demonstrated (Brommer, 1972). DBA/2 mice exhibited a more prolonged pattern of survival, but all mice were dead at 90 days of infection. Until that time point, a similar pattern was observed for BD_2 +/+ hybrids, which are of DBA/2 parental origin. However, 44% of these mice were still alive at 125 days, when the experiment was terminated. Apart from splenomegaly (1.4 ± 0.6 g), peripheral blood differentials and histological sections (lymph node, thymus) failed to reveal any signs of lymphoid or myeloid leukemia in these mice.

An additional effect of the W mutation, expressed in 70% mortality of BD_2 hybrids within 40 days, can be demonstrated. This may be due to an effect of Rauscher infection on increasing the severity of anemia, already present in these mice (Tables 3.1 and 3.3). Nevertheless, survival of the remaining mice followed a similar pattern as their +/+ littermates.

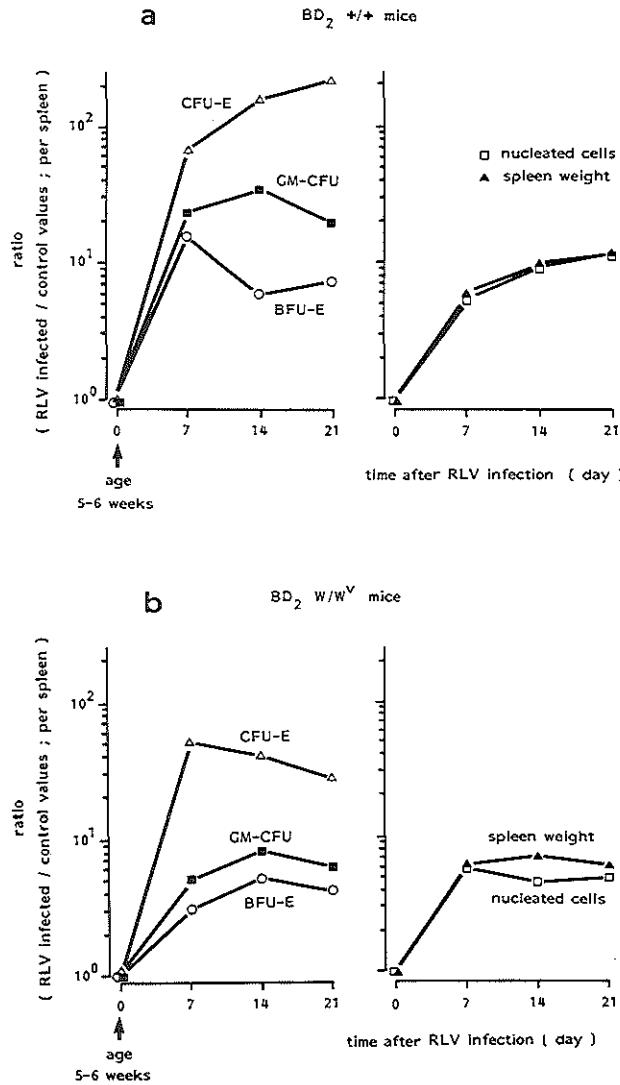


Figure 3.2:
Increase in haemopoietic progenitors in the spleen of RLV-infected mice.
Mice received 0.4 ml cell free extract, i.p., at day 0. Three mice were killed for each time point. The ratio at day of infection is 1. Control values of splenic CFU were determined on corresponding days, with the ratio calculated from the overall average values over the entire experiment, performed twice. A: BD +/+ control values for CFU-E, GM-CFU and BFU-E were 145 ± 23 x 10^3, 5.8 ± 1.3 x 10^3 and 10.3 ± 1.1 x 10^3, respectively. The number of cells per spleen was 1.7 ± 0.2 x 10^8, the spleen weight 0.11 ± 0.01 g.
B: Increase in haemopoietic progenitors in th spleen of RLV-infected BD_2-W/W^V mice. BD_2-W/W^V control values for CFU-E, GM-CFU and BFU-E were 183 ± 26 x 10^3, 10.4 ± 1.9 x 10^3 and 12 ± 1 x 10^3, respectively. The number of cells per spleen was 1.9 ± 0.3 x 10^8; the spleen weight was 0.12 ± 0.03 g.

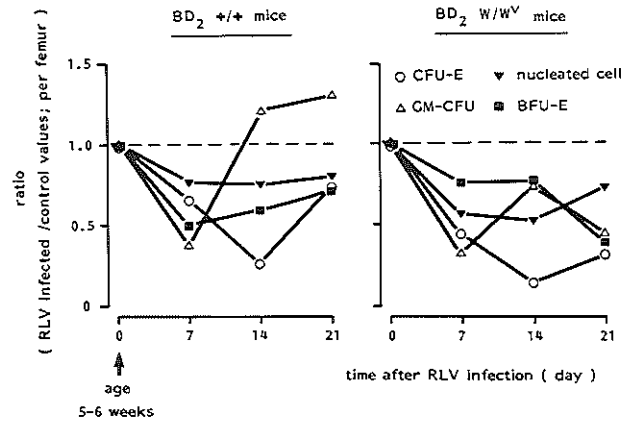


Figure 3.3:
Haemopoietic progenitor cells in the femur of RLV-infected mice.
A. BD_2 +/+ femoral CFU-E, GM-CFU and BFU-E values were 40.1 ± 6.2 x 10^3, 11.2 ± 1.2 x 10^3 and 6.4 ± 0.9 x 10^3, respectively. The femoral cell number was 14 ± 1.6 x 10^6.
B. BD_2-W/W^V femoral CFU-E, GM-CFU and BFU-E values were 39.0 ± 4.4 x 10^3, 20.4 ± 1.5 x 10^3 and 5.1 ± 0.7 x 10^3, respectively. The femoral cell number was 14.2 ± 1.6 x 10^6.

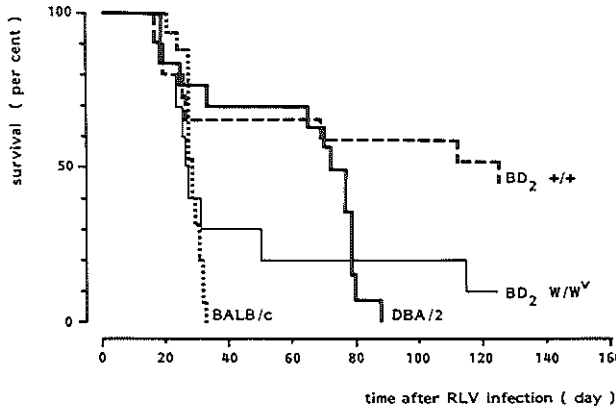


Figure 3.4:
Mortality of mice following RLV infection. Female mice aged 5-6 weeks were administered intraperitoneally with 0.4 ml cell free extract. The median survival time of BD_2 +/+ (n = 20), DBA/2 (n = 18) and BALB/c (n = 15) strains were 120, 72 and 29 days, respectively. The median survival time of BD_2-W/W^V genotypes (n = 20) is 27 days.

Strain-specific diversity in mortality has recently been documented in studies with Friend Murine Leukemia Virus (F-MuLV) as well (Chesebro et al., 1983). Although extensively investigated, no conclusive explanation for these observations could be found. A complex phenomenon, probably determined by various host genetic factors, of which only a few have been defined (Pincus, 1980), the cause of strain-specific death following RLV infection was not further pursued in this study.

3.2. MACROSCOPIC SPLEEN COLONY FORMATION FOLLOWING RAUSCHER INFECTION OF W/W^V MICE

The data presented in the previous section demonstrate that W/W^V mutants of susceptible mouse strains are capable of responding to RLV infection as well, as demonstrated by characteristic splenic enlargement, aberrant haematological findings, alterations in progeny production in the spleen and femur, as well as strain specific mortality.

In view of the highly deleterious effect of the W mutation on the generation of erythroid progeny in the spleen by stem cells (Wagemaker and Visser, 1979; Harrison and Russell, 1972; Gregory and Eaves, 1978) and the major involvement of the erythroid compartment in the prominent pathological features of Rauscher disease, the susceptibility of these mice to RLV infection may relate to the two major concepts regarding the primary target of this virus in the following manner:
a) the stem cell is not primarily involved in the development of Rauscher disease and erythroid precursors are the major viral targets; b) the stem cell is directly involved in the development of Rauscher erythroblastosis. In this case, an alteration in the capacity of W/W^V stem cells to differentiate in the spleen, must have occurred.

These alternative possibilities were tested by examining whether spleen or marrow cell suspensions from RLV infected W/W^V mice were capable of producing macroscopically visible colonies in the spleens of lethally irradiated recipients.

3.2.1. Morphological and histological evaluation

For this series of experiments, cell suspensions from either spleens or femurs of DBA/2-W/W^V mice, infected 21 days previously with RLV, were injected into lethally irradiated (9.25 Gy gamma rays) DBA/2 +/+ recipients. The spleen colony assay was performed as described in 2.4.

The presence of macroscopically visible nodules on the spleen of a recipient mouse transplanted with 5×10^6 spleen cells from RLV-infected W/W^V mice, is shown in Figure 3.5. A similar phenomenon was observed when marrow cell suspensions were used (see section 3.2.3). The normal gross morphological appearance of these colonies is clearly evident.

In order to assess the possibility that the colonies observed were host viral-induced spleen foci, two control groups of mice (6 mice per group) were given either a) the same spleen cell suspension, irradiated with 10Gy gamma rays prior to injection, or b) virus alone. No colonies were observed in recipient spleens of either group.

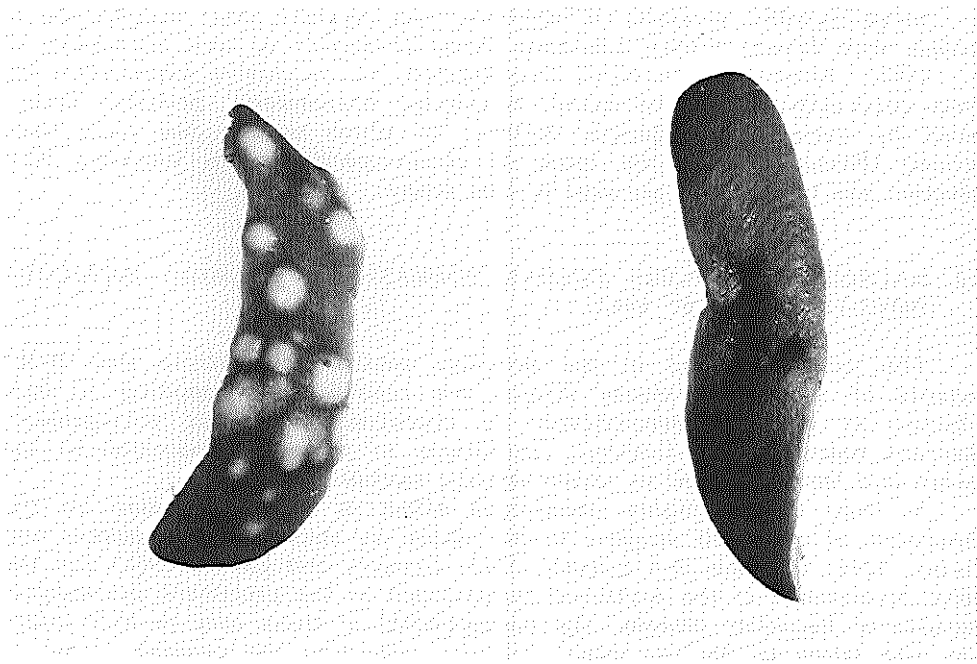


Figure 3.5:
Macroscopic spleen colony formation by RLV-infected W/W^V stem cells.

Several of the spleens were sectioned and stained for histological evaluation. The colonies observed appeared normal by this criterion as well (Figure 3.6). The macroscopic colonies were found to contain predominantly erythroid cells, although granulocytic, megakaryocytic or undifferentiated cell types could be detected as well. Mixed colonies containing megakaryocytes and erythroid cells were also observed. These findings contrast with the undifferentiated colony types produced by Rauscher transformed, pro-erythroblast-like cells (de Both et al., 1978a). The direct relationship between the number of cells injected and the number of colonies scored tens days thereafter, thus reinforcing their clonal nature, is shown in Figure 3.7.

Spleen or marrow cell suspensions from RLV-infected C57BL-W/W^V mice, which failed to develop Rauscher erythroblastosis, were incapable of producing macroscopic spleen colonies in lethally irradiated hosts. However, colony formation by RLV-infected spleen cells of BD_2-W/W^V mice could be detected in SFFV-resistant, irradiated C57BL hosts (Table 3.4). The lower number of CFU-S observed in lethally irradiated C57BL recipients of either +/+ or W/W^V spleen cells is attributable to the phenomenon of "genetic resistance" described for this donor/recipient combination (McCulloch and Till, 1963).

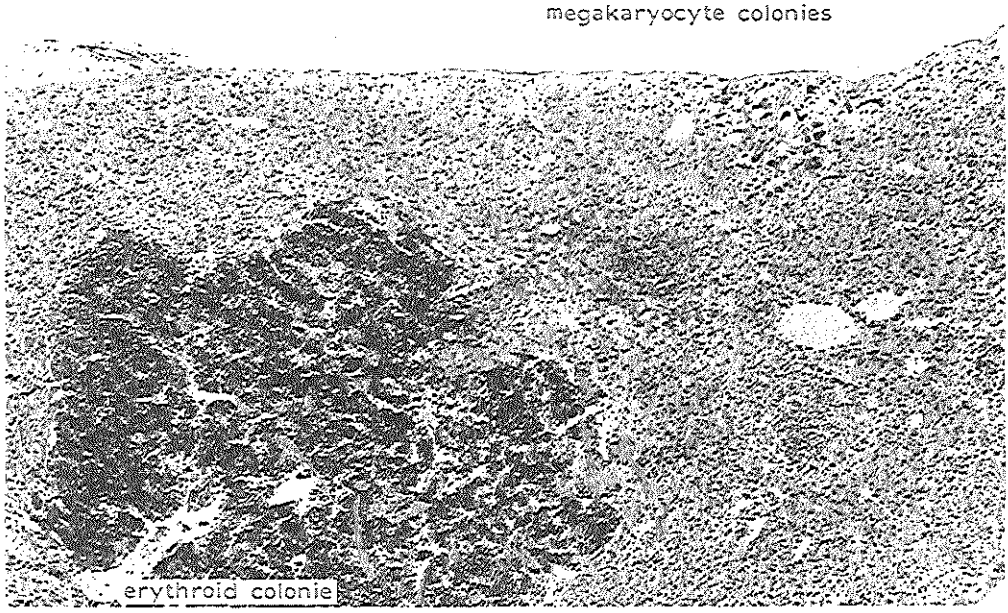


Figure 3.6:
Histology of spleen containing RLV-infected W/W^V CFU-S.

3.2.2 Generation of progeny by RLV-infected W/W^V CFU-S

Histological evaluation of spleen sections of mice transplanted with RLV-infected W/W^V haemopoietic cells (3.2.1) can only provide information as to the type of progeny present within the spleen colonies. The possibility that the formation of macroscopic spleen colonies following RLV infection of W/W^V mice was due to an excessive production of erythroid cells, was evaluated by comparing the number of erythroid (BFU-E, CFU-E) and granulocyte-macrophage (GM-CFU) progeny generated by either RLV-infected W/W^V or control +/+ CFU-S. The results of this analysis are shown in Table 3.5. In spite of the large differences in CFU-S frequency between the grafted cell suspensions, the number of progenitors generated per spleen colony by W/W^V and +/+ cells is strikingly similar. These findings are in accordance with the normal gross morphological appearance of spleen colonies formed by RLV-infected W/W^V stem cells (Figure 3.5).

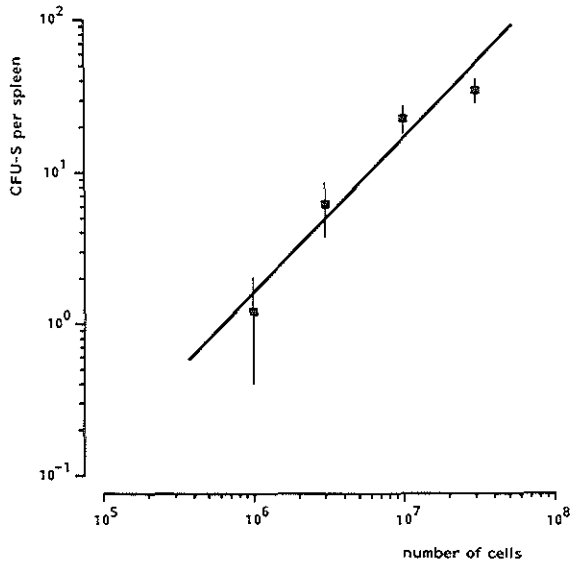


Figure 3.7:
Number of macroscopic spleen colonies by RLV-injected W/W^V spleen cells versus cell number injected.

Table 3.4

MACROSCOPIC SPLEEN COLONY FORMATION BY RLV-INFECTED BD_2-W/W^V STEM CELLS IN SFFV-RESISTANT HOSTS

spleen cell donor	CFU-S per 10^7 cells (± S.D.) DBA/2 ($FV2^{ss}$)	C57BL ($FV2^{rr}$)
BD_2-W/W^V RLV	3.2 ± 0.5	1.5 ± 0.2
BD_2 +/+	80 ± 8	42 ± 3

2 x 10^7 and 6 x 10^7 spleen cells from W/W^V-RLV mice, or 1.5 x 10^6 and 4.5 x 10^6 spleen cells from BD_2 +/+ mice, were injected into groups (n = 10) of lethally irradiated DBA/2 or C57BL recipients, respectively.

Table 3.5

GENERATION OF HAEMOPOIETIC PROGENITORS IN THE SPLEEN OF LETHALLY IRRADIATED MICE BY RLV-INFECTED W/W^V CFU-S
(day 9 post-transplantation)

marrow cell donor	number of cells injected	colonies per spleen ± SD	cells per colony*** ($\times 10^6$) ± SD	progenitors per colony ($\times 10^2$) ± SD		
				CFU-E	GM-CFU	BFU-E
W/W^V-RLV*	10^7	22 ± 6	1.5 ± 0.4	94 ± 25	4.3± 1.1	1.3± 0.3
BD_2 +/+	3×10^5	61 ± 13**	2.0 ± 0.4	68 ± 12	2.7± 0.3	0.4± 0.1

Lethally irradiated (9.0 Gy) BD_2 W/+ or W^V/+ littermates were used as recipients.
* BD_2-W/W^V mice - 3 weeks post RLV infection.
** The number of CFU-S was determined by spleen colony enumeration in mice (n = 8) which received 5×10^4 marrow cells of the same cell suspension.
*** The number of cells per colony was calculated following the subtraction of spleen cell numbers obtained in lethally irradiated, untransplanted controls.
Data represent a mean of 3 identical experiments.

3.2.3. Frequency of CFU-S

The frequency of CFU-S in both femur and spleen of RLV-infected W/W^V mice was examined at several time points after viral administration (Table 3.6).

The CFU-S frequency in both spleen and femur remains relatively constant throughout the observation period, suggesting that these values are a valid estimate of stem cell frequency in both haemopoietic organs. The formation of macroscopic spleen colonies in lethally irradiated W/W^V recipients, reinforces their W/W^V origin.

The CFU-S frequency is approximately 4% of normal in the spleen and 0.8% (BD_2-W/W^V) or 2% (DBA/2-W/W^V) in the femur. These values are within the same order of magnitude as the estimation obtained by comparative enumeration of microcolonies in histological sections of recipient spleens of W/W^V and +/+ marrow (Lewis et al., 1967; van Bekkum and Dicke, 1972).

3.2.4. Seeding efficiency of RLV-infected W/W^V stem cells

Estimation of the seeding efficiency of RLV-infected W/W^V stem cells was prompted by the low frequency of CFU-S observed (Tables 3.4-3.6).

The f-factor, indicating the fraction of stem cells (CFC-S) producing spleen colonies (CFU-S), was determined by enumerating the number of CFU-S recovered 2 hours postgrafting in spleens of recipients which had been irradiated 3 days previously, as modified by Lahiri et al. (1970). The results of this assay, performed as described in 2.4.2, are given in Table 3.7. The values obtained fail to demonstrate any effect of the W mutation or RLV infection on the seeding fraction of stem cells in the spleen. This is in contrast to the observations of OKunewick and Phillips (1973), who found a reduction in the seeding of RLV infected stem cells. These investigators, however, did not use preirradiated mice for estimation of CFU-S recovery 2 hours after transplantation, thus making their single time point determination inadequate for obtaining sufficiently reliable results. Variations due to splenic shrinkage after irradiation may lead to errors in determination of the seeding fraction, f, using this experimental procedure (Lahiri et al., 1970; Testa et al., 1972; Lord and Hendry, 1973; Van Bekkum, 1977).

Table 3.6

FREQUENCY OF CFU-S IN RLV-INFECTED W/W^V MICE

time after infection (weeks)	spleen		femur	
	DBA/2-W/W^V CFU-S per 10^7 cells ± SD	BD2-W/W^V	DBA/2-W/W^V CFU-S per 10^6 cells ± SD	BD2-W/W^V
1	14 ± 17	6.4 ± 0.7	3 ± 1.0	1.6 ± 0.3
2	n.d.	3.5 ± 0.5	n.d.	1.6 ± 0.4
3	13.2 ± 2	5.7 ± 0.5	6 ± 1.4	2.5 ± 0.7
3a*	24 ± 5	n.d.	n.d.	n.d.
W/W^V control	0	0	0	0
+/+ control**	388 ± 36	136 ± 12	229 ± 28	243 ± 23

Lethally irradiated DBA/2 (9.25 Gy gamma rays) or BD_2-W/+ and BD_2-W^V/+ (9.0 Gy gamma rays) mice were transplanted with 10^7 spleen or $2^2 \times 10^6$ marrow cells from W/W^V mice, or with 10^6 spleen or 5×10^4 marrow cells from +/+ controls. Spleen colony formation was determined as described as described in 2.4.

*Lethally irradiated (9.0 Gy γ-rays) DBA/2-W/W^V mice were used as recipients.

**Parental DBA/2 or BD_2 +/+ littermates of similar sex and age were used as controls.

3.2.5. Specificity of W/W^V stem cell repair

The question as to whether formation of macroscopic spleen colonies by RLV-infected stem cells of W/W^V mice is a direct consequence of the anemic state following infection with the virus, was examined by exposure of these mice to Bacillus-Calmette Guèrin (BCG) and Corynebacterium parvum, known to induce anemia characterized by active splenic erythropoiesis, which includes an increase in the number of CFU-S in the spleen (Toujas et al., 1972; Brozovic et al., 1975; Marchal et al., 1981). In addition, the effect of the erythroblastosis inducing polycythemic strain of the Friend virus complex (Mirand, 1967), was examined.

For this purpose, three separate groups, containing 4 BD_2-W/W^V mice each, were given either of the following:

a) killed Corynebacterium parvum (Wellcome, England), 0.35 mg per mouse, by intravenous administration;

b) BCG (RIV batch 077, Kreeftenburg et al., 1981), 5×10^6 culturable particles per mouse, administered intravenously;

c) N-B tropic pseudotype of the polycythemic strain of the Friend virus complex (FLV-P), 0.2 ml cell free extract, administered intraperitoneally.

Table 3.7

DETERMINATION OF THE SPLENIC SEEDING FRACTION f OF RLV-INFECTED STEM CELLS

cells injected	number of cells injected	CFC-S injected ± SD*	total CFU-S recovered from spleens 2 hrs after transplantation** ± SD	seeding fraction f ± SD
+/+ marrow	1×10^7	3233 ± 327	50.0 ± 7.7	0.0155 ± 0.002
+/+ RLV marrow	1×10^7	3900 ± 239	46.0 ± 5.2	0.0118 ± 0.002
+/+ RLV spleen	4×10^7	700 ± 55	11.0 ± 2.3	0.0157 ± 0.003
W/W^V RLV marrow	2×10^7	180 ± 19	2.7 ± 0.9	0.0150 ± 0.006
W/W^V RLV spleen	1×10^8	49 ± 2.3	0.8 ± 0.56	0.0163 ± 0.01

Lethally irradiated (9.0 Gy) BD_2 W/+ or W^V/+ littermates were used as recipients.

* The number of CFU-S injected was determined in a separate group of recipients which had received appropriate dilutions of the injected cell suspensions.

**The total number of CFU-S recovered was calculated according to the fraction of spleen injected into secondary irradiated recipients.

Three weeks later these mice were killed and their spleen weights measured. Cell suspensions from pooled spleens or femurs were injected into groups of lethally irradiated recipients for assessment of macroscopic spleen colony formation. The results of these experiments are shown in Table 3.8. The effect of these agents on inducing significant splenic enlargement is clearly evident. Nevertheless, spleen colony formation was found only following RLV and FLV-P infection of BD_2-W/W^V mice, whereas both BCG and C. parvum were ineffective at inducing this phenomenon. Although a comparatively lower increase in splenic weight was induced by the latter, this factor is not directly relevant for macroscopic spleen colony formation; spleen weights of DBA/2-W/W^V mice 1 week after RLV infection (Table 3.6) were 0.29 ± 0.05 grams.

These findings demonstrate that the repair of W/W^V stem cell phenotypic expression cannot be induced by any nonviral agent which provokes active haemopoiesis in the spleen. Neither is this phenomenon related to anemia, as shown by the FLV-P group. Rather, an effect at the level of the genome, mediated by proviral integration, seems to be implied by these data.

Table 3.8

SPECIFICITY OF THE "REPAIR" OF W/W^V STEM CELLS BY RAUSCHER VIRUS

agent	spleen weight (g) ± SD	haematocrit (%) ± SD	spleen colonies spleen$_7$ (per 10^7 cells) ± SD	spleen colonies femur$_6$ (per 10^6 cells) ± SD
RLV	1.1 ± 0.5	20 ± 4	5.7 ± 0.5	2.5 ± 0.7
FLV-P	1.03 ± 0.4	38 ± 5	5.1 ± 0.8	1.6 ± 0.6
BCG	0.44 ± 0.07	23 ± 4	-	-
C. parvum	0.58 ± 0.03	24 ± 5	-	-
W/W^V control	0.12 ± 0.03	30 ± 6.8	-	-

Recipient mice and cell numbers injected as in legend to Table 3.4. Determined three weeks post-infection.

3.3 CONCLUSIONS

Infection of W/W^V mice with Rauscher Leukemia Virus has failed to demonstrate any major effect of the W mutation on the development of Rauscher disease and its characteristic symptoms. Using splenomegaly as a criterion, this has recently been confirmed by Ostertag et al. (1982).

W/W^V stem cells are characterized by their defective capacity to give rise to macroscopic spleen colonies in lethally irradiated hosts, a property which relates to their reduced differentiation within a splenic environment (McCulloch et al., 1964; Lewis et al., 1967; Bennett et al., 1968; Harrison, 1972). The development of Rauscher disease in W/W^V mutants is accompanied by a restoration of this defect, as evidenced by the spleen colony assay.

The alteration of W/W^V stem cell phenotypic expression by RLV infection is the first observation which demonstrates the direct involvement of the haemopoietic stem cell in the pathogenesis of Rauscher disease. That the W/W^V mouse offers a unique experimental system for detection of enhanced stem cell differentiation and proliferation in the spleen as a result of RLV infection, is clearly evident. The etiological link between this property and the development of Rauscher erythroblastosis is further supported by the resistance of C57BL W/W^V mice to both manifestations. The lesser extent of splenomegaly in RLV-infected susceptible genotypes of these mutants, is probably attributed to their reduced stem cell numbers.

The highly reduced number of stem cells in W/W^V mice is not a surprising observation. Sterility and absence of coat pigmentation in mice carrying the W mutation, have both been traced to a severe deficiency in production of primordial germ cells and melanoblasts during embryonic development (Mintz and Russell, 1957; Mayer and Green, 1968). The average stem cell frequency, approximately 40-fold lower than in normal mice, is within the same order of magnitude as the estimation obtained by enumeration of microcolonies in histological sections of spleens of irradiated recipients of W/W^V marrow grafts (Lewis et al., 1967; Van Bekkum and Dicke, 1972).

Although very little can presently be stated in relation to the mechanism(s) by which RLV infection enhances W/W^V stem cell differentiation, evidence that it is primarily related to a direct effect of the virus on stem cells themselves, rather than on the splenic environment, has been provided (Table 3.4). The restriction of this expression to mice carrying the $FV\text{-}2^S$ genotype, the capacity of Friend virus to induce macroscopic spleen colony formation by W/W^V stem cells, as well as the similarity between Friend and Rauscher SFFV components and their role in initiating erythroblastosis (Linemeyer et al., 1981, 1982; Ruta and Kabat, 1980; Machida et al., 1984), all strongly suggest a major role for the R-SFFV component in this phenomenon.

CHAPTER IV

FUNCTIONAL EXPRESSION OF RLV-INFECTED W/W^V STEM CELLS

Macroscopic spleen colony formation following Rauscher infection of W/W^V mice (Chapter 3) indicates a repair of their stem cell defect. In order to further establish these observations, however, additional studies of the nature of the cells which have been restored by RLV infection, must be performed.

Analysis of the capacity of W/W^V CFU-S to provide protection from lethal irradiation, is required for evaluation of the relationship between macroscopic spleen colony forming cells and functional pluripotential stem cells. These studies are of special relevance in view of recent observations suggesting that not all spleen colonies are indeed derived from pluripotent, haemopoietic stem cells (Abramson et al., 1977; Magli et al., 1982; Baines and Visser, 1983).

Both the macrocytic anemia of W/W^V mice (Russell and Bernstein, 1966), as well as the complete absence of tissue mast cells (Kitamura et al., 1978), have been attributed to defective W/W^V stem cell expression. Evaluation of the effect of RLV infection on these properties may provide additional information as to the extent to which the expression of these cells is restored by the virus.

4.1 30-DAY RADIOPROTECTIVE CAPACITY OF RLV-INFECTED W/W^V CFU-S

The radioprotective capacity of macroscopic spleen colony forming cells of RLV-infected W/W^V mice was analyzed in a comparative 30-day survival assay, using haemopoietic cell suspensions from RLV-infected BD_2-W/W^V or control uninfected BD_2-+/+ mice.

Lethally irradiated (9.0 Gy) BD_2-W/W^V mice were used as recipients. These were injected intravenously with 0.5 ml of spleen cell suspensions containing 5×10^6, 10^7, 3×10^7 and 9×10^7 cells from RLV-infected BD_2-W/W^V donors of the same sex. The spleen served as a practical source of stem cells since by its enlargement due to RLV infection it contained a larger number of CFU-S than the femur (Table 3.6), thus reducing the number of donor W/W^V mice required. Furthermore, previous studies had shown that the 30-day radioprotective capacity of spleen cells is unaltered by RLV infection (Brommer, 1972; Brommer and Bentvelzen, 1974). In view of reported differences between the radioprotective capacity of splenic and femoral CFU-S (Kretchmar and Conover, 1970), the spleen was used as a stem cell source from uninfected BD_2 +/+ mice, as well. 5×10^5, 1×10^6, 2×10^6 and 4×10^6 spleen cell suspensions from these donors were injected

into lethally irradiated BD_2-W/W^V recipients. The number of surviving mice in each experimental group was scored 30 days after transplantation. The survival in relation to CFU-S numbers injected is illustrated in Figure 4.1.

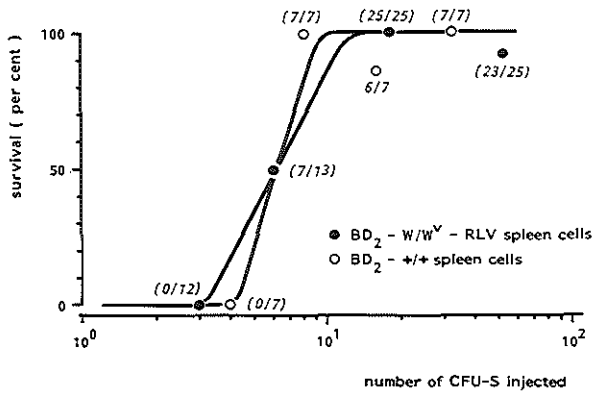


Figure 4.1:
30-day survival of lethally irradiated (9.0 Gy) BD_2-W/W^V mice after transplantation of RLV-infected BD_2-W/W^V CFU-S. The numbers in parentheses represent the actual number of surviving mice per number of mice evaluated. The CFU-S frequency was 6 ± 2 for 10^7 spleen cells of RLV-infected BD_2-W/W^V mice and 8 ± 1.3 for 10^6 BD_2 +/+ spleen cells.

As clearly demonstrated, the number of CFU-S required for 50% protection of lethally irradiated BD_2-W/W^V mice was essentially the same (6 ± 2 for RLV-infected W/W^V spleen cells versus 5 ± 2 for +/+ spleen cells), thus reinforcing the identity between macroscopic spleen colony forming and pluripotent haemopoietic stem cells, under these exceptional circumstances.

The total number of RLV-infected W/W^V spleen cells required for 50% radioprotection was approximately 10-fold higher than that of BD_2 +/+ mice, in accordance with the reduced concentration of stem cells in this mutant (Lewis et al., 1967; Van Bekkum and Dicke, 1972, Chapter 3). These findings are also in accordance with Harrison's observations of a 10-fold lower radioprotective capacity of W/W^V marrow (Harrison, 1972).

4.2 GENERATION OF CIRCULATING ERYTHROCYTES

Transplantation of W/W^V mice with stem cells from normal donors cures them of their macrocytic anemia. The cure is manifested by an increase in hematocrit and peripheral red blood cell count and by complete replacement of macrocytic erythrocytes with mature red blood cells of normal volume and size distribution, within 45 days after transplantation (Russell et al., 1956; Lushbaugh and Russell, 1963; Wiktor-Jedrzejczak et al., 1979).

The use of BD_2-W/W^V mice as lethally irradiated recipients in the 30 day survival studies (4.1) enabled assessment of both the numerical and physical characteristics of mature erythroid progeny generated by RLV-infected W/W^V stem cells, without any "background interference" which would have been present in normal +/+ recipients of such a graft.

Analysis of peripheral red blood cell haematology of lethally irradiated BD_2-W/W^V mice reconstituted with RLV-infected W/W^V spleen cells was performed. Figure 4.2 shows a representative red blood cell size distribution profile 6-7 weeks after transplantation, in comparison to that of untransplanted or +/+ littermate controls. These profiles clearly show that the W/W^V recipients have been reconstituted with erythrocytes of normal size distribution and mean corpuscular volume (MCV). A numerical representation of red blood cell haematology in reconstituted BD_2-W/W^V mice is shown in Table 4.1. The normalization in MCV is clearly evident. A significant ($p < 0.01$) increase in red cell numbers can also be demonstrated. These are comparable to the values obtained in W/W^V mice reconstituted with +/+ and +/+ RLV spleen cells.

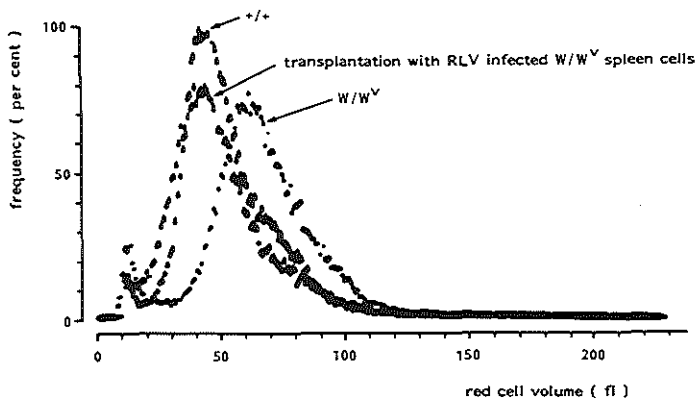


Figure 4.2:
Red blood cell size distribution profile of lethally irradiated BD_2-W/W^V mice transplanted 6-7 weeks previously with RLV-infected W/W^V spleen cells. Performed as described in Chapter 2.8.2.

Table 4.1

RED BLOOD CELL HAEMATOLOGY OF LETHALLY IRRADIATED BD_2-W/W^V MICE RECONSTITUTED WITH RLV INFECTED SPLEEN CELLS
(6-7 weeks post-transplantation)

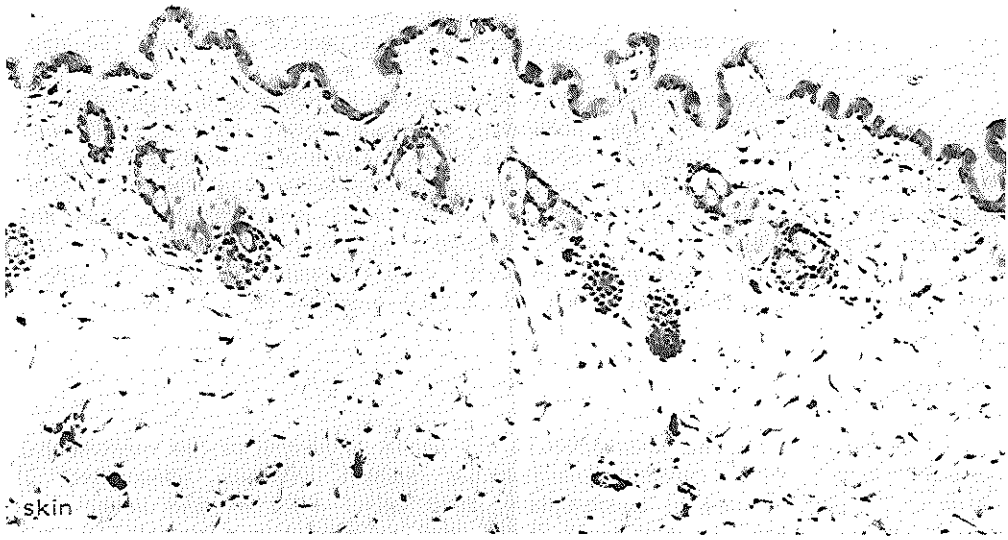
genotype	treatment	erythrocytes ($\times 10^9.ml^{-1}$) ± SD	mean corpuscular volume (fl)* ± SD	haematocrit (%) ± SD
W/W^V (n=6)	infected 3 weeks previously with RLV	3.3 ± 1.2	57 ± 1.9	20 ± 4.0
W/W^V (n=9)	none	4.4 ± 1.3	60 ± 5.5	30 ± 6.8
W/W^V (n=7)**	transplantation with RLV-infected W/W^V spleen cells	7.3 ± 0.6	42 ± 2.6	31 ± 3.7
W/W^V (n=7)**	transplantation with +/+ spleen cells	8.3 ± 1.7	47 ± 2.1	40 ± 10
W/W^V (n=2)**	transplantation with RLV-infected +/+ spleen cells	8.1 (7.5, 8.6)	42 (41, 43)	34 (30, 37)

*calculated as the ratio between haematocrit and red cell count.
**mice were transplanted with equivalent numbers of RLV infected W/W^V, +/+ and +/+-RLV CFU-S (27, 24 and 30, respectively).

4.3 GENERATION OF TISSUE MAST CELLS

The populations of both mucosal and connective tissue mast cells in W/W^V mice are either completely absent or highly deficient (less than 1% of normal in skin, for example) (Kitamura et al., 1978; Crowle, 1982). Transplantation of marrow or spleen cells from +/+ donors corrects this deficiency and the common origin of tissue mast cells, granulocytes and erythrocytes in the spleen colony forming cell (CFU-S) was demonstrated in W/W^V mice reconstituted with cells derived from individual spleen colonies (Kitamura et al., 1981).

Since the rate of appearance of tissue mast cells is slow, reconstituted mice are usually evaluated for the presence of these cells 15 weeks after transplantation (Kitamura et al., 1977, 1978). Therefore, long term surviving



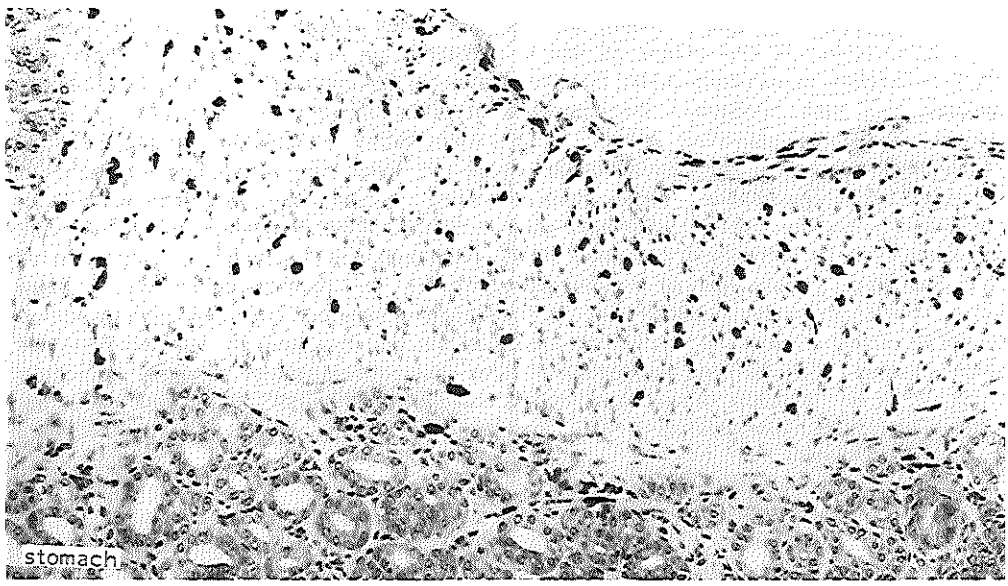


Figure 4.3:
The presence of mast cells in the skin and stomach of a lethally irradiated W/W^V mouse reconstituted with RLV-infected W/W^V stem cells.

BD_2-W/W^V mice reconstituted with either W/W^V RLV infected spleen cells (n = 15) or control +/+ littermate spleen cells (n = 4) were sacrificed 150 days after transplantation.

Pieces of dorsal skin and forestomach were removed and processed for histological evaluation of the presence of mast cells, by staining with Toluidine blue (2.8). Although repair of all recipients was confirmed by MCV evaluation, only one of the 15 mice reconstituted with RLV infected W/W^V spleen cells (6×10^7) was found to contain mast cells in both skin and stomach. Their presence was clearly evident, as shown in Figure 4.3. The same phenomenon was apparent in all W/W^V mice transplanted with +/+ spleen cells (5×10^6) from uninfected (n = 5) or RLV-infected (n = 2) mice.

4.4 CONCLUSIONS

The survival studies described in 4.1 serve to reinforce the relationship between W/W^V cells forming macroscopic spleen colonies (described in Chapter 3) and functional haemopoietic stem cells. The ten-fold lower than normal radioprotective capacity of W/W^V marrow cells (Harrison, 1972), can now be explained by the reduced frequency of stem cells in these mutants. Harrison's findings further demonstrate that although defective in spleen colony forming capacity, W/W^V stem cells are nevertheless capable of producing sufficient progeny which are required for effective radioprotection.

Elimination of the effect of the W mutation on erythrocyte production by RLV infection is evident both by the physical properties and the number of mature red cell progeny. Macrocytosis in this mutant is assumed to be a direct result of elevated levels of erythropoietin (Keighley et al., 1966), characterized by a proportional increase in red cell haemoglobin content (Russell and Bernstein, 1966) and elevated nucleoside deaminase levels in circulating erythrocytes (Harrison et al., 1975). The formation of normocytic erythrocytes in lethally irradiated W/W^V recipients is a direct consequence of the elevated generation of erythroid progeny by RLV-infected W/W^V stem cells. Both phenomena could not be detected following the primary infection of W/W^V mice (Table 4.1), which resulted in an increase in the severity of their anemia. The failure to detect all the characteristics of primary infection with RLV in lethally irradiated mice transplanted with RLV-infected stem cells, although unexplained, has been observed in BALB/c mice as well (Brommer, 1972).

Restoration of defective tissue mast cell generation by infection of W/W^V stem cells with Rauscher Virus, has been observed in one mouse. The absence of tissue mast cells in the majority of W/W^V mice reconstituted with RLV-infected W/W^V spleen cells may be due to the fact that more time is still required for the appearance of such cells in these mice. Even under circumstances in which W/W^V mice are transplanted with marrow cells from +/+ genotypes, the appearance of tissue mast cells has been shown to be a very slow process (Kitamura et al., 1977, 1978). The possibility that RLV infection in itself inhibits stem cell differentiation into tissue mast cells is ruled out by their presence in controls which had received spleen cells from RLV-infected +/+ donors.

CHAPTER V

DEVELOPMENT OF SERUM-FREE CULTURES FOR THE GROWTH OF MURINE HAEMOPOIETIC CELLS

Culture systems for the growth of haemopoietic cells in vitro can provide useful tools for evaluation of the role of regulatory mechanisms in the expression of haematological disorders. To date, such investigations have mainly been approached by analysis of the sensitivity of haemopoietic cells demonstrating aberrant growth, to the specific haemopoietic factors which stimulate their proliferation and maturation in vitro (Liao and Axelrad, 1975; Hasthorpe and Bol, 1979; Gallicchio and Murphy, 1981; Kreja and Seidel, 1981; Klein et al., 1981; Ostertag and Pragnell, 1981; Fagg et al., 1983; Hankins, 1983). Studies related to the effect of Rauscher infection on regulator sensitivity, however, have provided conflicting results (Hasthorpe and Bol, 1979; Gallicchio and Murphy, 1981; Walma and Wagemaker, 1979; Ostertag and Pragnell, 1981). Thus, in order to accurately investigate the responsiveness of RLV-infected haemopoietic cells from either normal or genetically anemic W/W^V mice to specific factors in vitro, any possible contamination of such regulators within the culture system, must first be eliminated. Since one source of such activities is serum (Iscove, 1978; Wagemaker, 1978), commonly used to supplement synthetic media in which mammalian cells are grown, the development of serum free cultures for the growth of murine haemopoietic cells, was initiated.

Due to its complex nature, serum provides the cells with a large variety of uncontrolled and undefined components, whose nutritional value is difficult to evaluate. The complete replacement of serum with chemically defined substances, may thus also yield information as to the nonspecific growth requirements of haemopoietic cells of various lineages and stages of differentiation. The development of defined, serum free cultures for colony formation by granulocyte-macrophage and erythroid progenitors, as well as of replicating stem cells stimulated by SAF, is described in this chapter.

5.1 GRANULOCYTE-MACROPHAGE PROGENITORS (GM-CFU)

The formation of colonies consisting of granulocytes and/or macrophages can be induced by stimulation with several types of colony stimulating factors (Burgess and Metcalf, 1981). Two distinct types of such molecules have been purified to apparent homogeneity. M-CSF, which stimulates preferentially the formation of colonies consisting of macrophages, has been purified as a 70,000 dalton glycoprotein from L-cell conditioned medium (Stanley and Heard, 1977) and as a 61,000 glycoprotein from pregnant mouse uteri extract or PMUE (Brouwer et al., 1980). GM-CSF, purified from mouse

lung conditioned medium as a 23,000 dalton MW glycoprotein (Burgess et al., 1977), stimulates the formation of a high proportion of granulocytic or mixed granulocyte- macrophage colonies at high concentrations and mainly macrophage colonies at low concentrations. GM-CSF is also present in supernatants of lectin-stimulated mouse spleen cells (Burgess et al., 1978; Wagemaker and Burger, 1982). Evidence that M-CSF and GM-CSF share a common population of target cells comes from the overlapping buoyant density distribution of GM-CFU stimulated with purified preparations of either regulator (Williams and Eger, 1978), the nonadditive effect of these regulators on colony formation in culture (Metcalf and Burgess, 1981), as well as direct evidence for the existence of GM-CFU responding to both CSF types (Metcalf and Burgess, 1981).

Murine haemopoietic progenitors of the granulocyte-macrophage as well as erythroid lineages can be conventionally grown in methylcellulose cultures consisting of a low fetal calf serum concentration (2-5%), if supplemented with lecithin, bovine serum albumin, iron-saturated transferrin and selenite (Guilbert and Iscove, 1976; Wagemaker and Visser, 1980; described fully in 2.5.1). The complete elimination of serum from cultures of late erythroid progenitors (CFU-E), achieved by the addition of a sonicated lipid mixture consisting of cholesterol, phospholipid and unsaturated fatty acid (Iscove et al., 1980), prompted the analysis of the lipid requirements for serum free growth of granulocyte- macrophage progenitors, as well. Due to the ready availability of highly purified preparations of $M\text{-}CSF_{pmue}$ at the initiation of this study, the clonal assay for detection of colony formation by M-CSF stimulated GM-CFU, was used.

Table 5.1

GM-CFU NUMBER RELATED TO FETAL CALF SERUM CONCENTRATION

percentage fetal calf serum	number of colonies per 10^5 cells*
5	83 ± 9
4	82 ± 5
3	80 ± 9
2	76 ± 8
1	51 ± 7
0.5	42 ± 7
0	0

BCBAF1 females, aged 8-12 weeks, were used throughout all the experiments described in this section.
Cultures consisted of modified Dulbecco's medium, supplemented with 1% bovine serum albumin, 1×10^{-6} M Fe-saturated transferrin, 10^{-7} M selenite, 10^{-4} M mercaptoethanol, 10^{-3} $g.l^{-1}$ nucleosides and 20 $\mu g.ml^{-1}$ egg lecithin (2.5.1)

The effect observed upon reduction of the fetal calf serum concentration below 2% in conventional, lecithin supplemented cultures, is shown in Table 5.1. These data demonstrate that lecithin alone cannot support serum free growth of GM-CFU. This was confirmed for several concentrations of lecithin, as shown in Table 5.2. Any possible inhibition due to contaminants present in the crude egg phospholipid preparation, is ruled out by the identical results obtained with synthetic lecithin. Figure 5.1 demonstrates the effect of adding cholesterol and linoleic acid to cultures devoid of serum. Cholesterol alone was found to support a substantial number of colonies. Whereas linoleic acid was insufficient alone at replacing the requirement for serum for colony formation, its addition to the cultures was beneficial in the presence of cholesterol, resulting in further augmentation of colony numbers. The optimal concentration of linoleic acid for this synergistic effect, was found to be limited to a narrow range within an equimolar ratio for both lipids. The data of Table 5.3 demonstrate the markedly inhibitory effect upon the addition of increasing phospholipid concentrations to serum free cultures supplemented with an optimal concentration (1.5×10^{-5} M) of cholesterol and linoleic acid. This effect was not observed in control cultures supplemented with fetal calf serum (Tables 5.2 and 5.3).

Table 5.2

MACROPHAGE COLONY FORMATION
IN PHOSPHOLIPID SUPPLEMENTED CULTURES

lipids		colonies/10^5 cells	
		serum free	serum supplemented[c]
egg lecithin	2.5 µg/ml	0	70 ± 8
	10 µg/ml	0	70 ± 8
	20 µg/ml	0	73 ± 9
	40 µg/ml	0	69 ± 8
synthetic lecithin[a]	2.5 µg/ml	0	66 ± 8
	10 µg/ml	0	62 ± 8
	20 µg/ml	0	77 ± 8
	40 µg/ml	0	70 ± 7
control - lipid[b]		0	70 ± 8

mean ± standard deviation of three individual experiments.
[a] L-alpha-lecithin (β-oleoyl, γ-palmitoyl)
[b] control cultures without lipid received 0.2% ethanol alone (see 2.5.1)
[c] 5% fetal calf serum

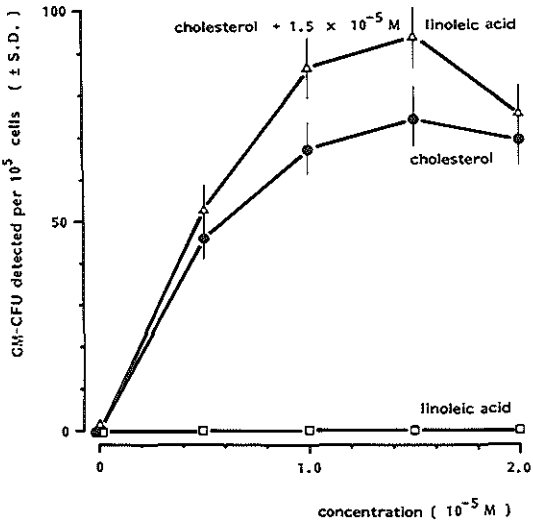


Figure 5.1:
Effect of cholesterol and linoleic acid on M-CSF stimulated macrophage colony formation in serum free cultures. Culture conditions are the same as described in Table 5.1, except for the omission of egg lecithin and fetal calf serum. Data represent the mean ± standard deviation of two independent experiments.

Table 5.3

THE INHIBITORY EFFECT OF LECITHIN ON GM-CFU DETECTION IN SERUM FREE CULTURES

		colonies/10^5 cells serum free	serum supplemented[c]
linoleic + cholesterol (1.5×10^{-5} M)		93 ± 10	77 ± 9
+ lecithin[a]	2.5 µg/ml	82 ± 9	73 ± 8
	10 µg/ml	43 ± 7	80 ± 9
	20 µg/ml	26 ± 5	79 ± 9
	40 µg/ml	16 ± 4	79 ± 9
control - fatty acids[b]		0	70 ± 8

a,b,c see legends to Table 5.2

With the lipid requirements for serum free growth of M-CSF stimulated mouse GM-CFU fully defined, any possible major contribution of cellular products to the detection of GM-CFU in the serum free culture system, was examined by evaluation of colony growth at various cell numbers plated. The data in Figure 5.2, demonstrating a direct relationship between both parameters, rule out this possibility. The dose response curve for purified M-CSF under serum free or serum-supplemented culture conditions, is illustrated in Figure 5.3. No change in the range of sensitivity to this regulator can be demonstrated. However, under saturating CSF levels, serum free cultures were found to promote an additional number of macrophage colonies (38 ± 7 per cent increase, mean of 10 experiments), colony size remaining unchanged.

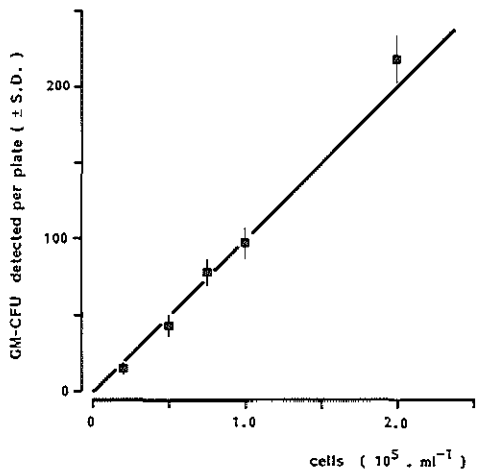


Figure 5.2:
Number of macrophage colonies enumerated versus cell number plated in serum free cultures supplemented with 1.5 x 10^{-5} M of cholesterol and linoleic acid.

Stimulation of GM-CFU in serum free cultures by purified GM-CSF revealed poor colony formation, which failed to reach colony size and numbers obtained in cultures supplemented with 5% fetal calf serum. Among a variety of components tested for their capacity to meet the additional requirements for GM-CSF stimulated colony formation, hydrocortisone, at a concentration of 10^{-6} M, proved to be effective at augmenting colony formation in cultures to values which slightly exceeded those of serum supplemented controls (Figure 5.4). Hydrocortisone slightly inhibited M-CSF stimulated colony formation. The colony types obtained in GM-CSF stimulated serum free cultures are given in Table 5.4.

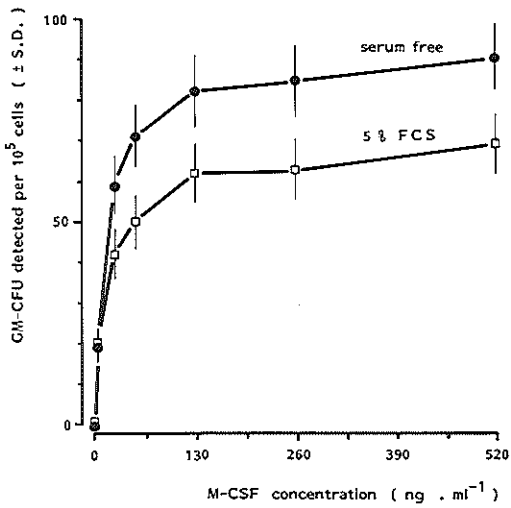


Figure 5.3:
Dose response curve for M-CSF with or without fetal calf serum.

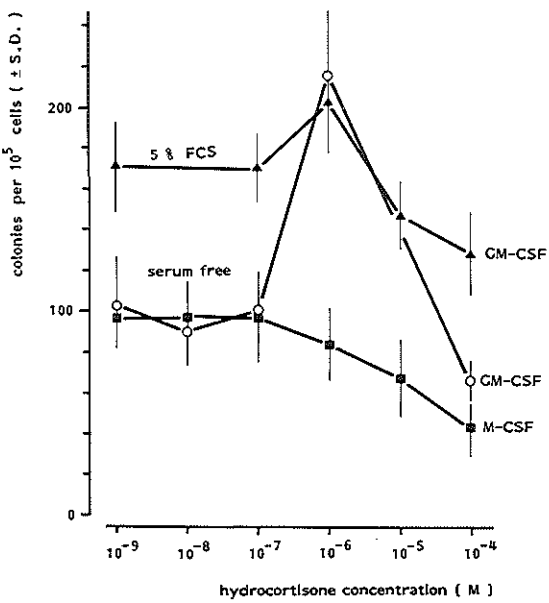


Figure 5.4:
Effect of hydrocortisone on colony formation in cultures stimulated with purified M-CSF or GM-CSF.

Table 5.4

COMPOSITION OF COLONIES IN GM-CSF-STIMULATED CULTURES

culture conditions	colonies per 10^5 cells	percentage of colonies			
		granulocyte	granulocyte/ macrophage	macrophage	undifferentiated
serum-free - hydrocortisone	77 ± 11	10 ± 2	-	-	90 ± 14
serum-free + hydrocortisone	165 ± 22	30 ± 6	26 ± 5	30 ± 6	13 ± 3
controls*	145 ± 17	34 ± 7	31 ± 6	39 ± 8	-

Colonies were typed at day 7 of incubation.
*controls contained 5% FCS - hydrocortisone.

Colonies representing successive stages of development within the granulocyte-macrophage lineage, have been described (Bol et al., 1979; Bol and Williams, 1980). Whereas M-CSF_{pmue}-stimulated colony formation detects a subpopulation of GM-CFU claimed to represent relatively mature progenitor cells, the stimulation of mouse marrow cells with preparations containing CSF-enhancing activity (CEA), enables the detection of colony formation by a low density subpopulation (Van den Engh and Bol, 1975; Bol et al., 1977; Williams and Eger, 1978; Bol et al., 1979), assumed to represent developmentally early progenitors of this lineage (Bol and Williams, 1980; Van Bekkum et al., 1979). Such factor(s), distinct from M-CSF and GM-CSF (Wagemaker, 1980a; 1981) do not themselves stimulate the formation of granulocyte and/or macrophage colonies, but further promote colony numbers in cultures already stimulated with a saturating dose of CSF (Van den Engh and Bol, 1975; Byrne et al., 1977; Wagemaker and Peters, 1978; Bol et al., 1979). They probably act by inducing the production of CSF-responsive cells by low density GM-CFU.

Enhancement of colony formation by CEA partially purified from medium conditioned by Concanavalin A stimulated mouse spleen cells, was examined in M-CSF stimulated serum free cultures. The results of this assay, as compared to serum supplemented controls, are given in Table 5.5. These data clearly demonstrate that the serum free cultures adapted for optimal colony formation by relatively mature precursors of the granulocyte-macrophage lineage, are appropriate for the detection of developmentally early progenitors, as well.

Table 5.5

ENHANCEMENT OF COLONY FORMATION BY CEA
IN SERUM-FREE CULTURES

	GM-CFU detected per 10^5 cells (± SD)	
	serum free	serum supplemented[c]
[a] CEA alone	9 ± 4	14 ± 5
[b] CSF alone	90 ± 13	82 ± 13
[b] CSF + CEA	150 ± 17	140 ± 17

[a]The source of CEA was a stage II preparation of medium conditioned by Con-A stimulated mouse spleen cells (MSCM), added at a concentration of 20 µl per ml.
[b]M-CSF was added at a saturating concentration of 4.7 $\mu g.ml^{-1}$
[c]5% fetal calf serum
Cultures were scored after 7 days of incubation.

5.2 ERYTHROID PROGENITORS (BFU-E)

The presence of specific haemopoietic regulators in certain batches of fetal calf serum used to supplement culture media for the growth of haemopoietic cells, was initially demonstrated in assays of developmentally early (BFU-E) erythroid progenitors (Iscove, 1978; Wagemaker, 1978). A 10-fold reduction in the amount of "selected" serum required to achieve a given cloning efficiency, could be obtained by the addition of a glycoprotein factor, termed burst forming activity (BFA), partially purified from 7-day supernatants of lectin-stimulated mouse spleen cells, in which it was found to be present (Iscove, 1978). The action of BFA was subsequently shown to be restricted to the early stages of differentiation within the erythroid compartment, which are succeeded by a gradual acquisition of sensitivity to erythropoietin (Iscove, 1978; Wagemaker, 1978, 1981). This was demonstrated by the capacity to delay the addition of erythropoietin to BFA-stimulated cultures for a period of up to 4 days, without affecting the number of bursts scored after 10 days of incubation (Iscove, 1978; Wagemaker, 1978).

Attempts to completely replace the serum requirements of erythroid progenitors have so far been only partially fulfilled. The successful proliferation and maturation of erythropoietin-dependent late erythroid precursors (CFU-E) in serum free cultures (Iscove et al., 1980), has already been mentioned. Under equivalent serum free culture conditions, however, only 20% of the maximal BFU-E obtained at 10% fetal calf serum, could be detected (Iscove, 1978). Studies of the nonspecific growth requirements of the developmentally early stages of erythroid differentiation, described in this

section, were performed in cultures in which the addition of erythropoietin was delayed for 4 days. This was of special importance in view of the relatively crude erythropoietin preparation used (2.7.4).

The specific lipid requirements for the initial stages of erythroid differentiation, have not been investigated. The results of a representative experiment in which the various lipids were analyzed for their capacity to promote serum free growth of BFU-E, is given in Table 5.6. These findings are identical to those obtained with M-CSF stimulated GM-CFU, demonstrating a major requirement for cholesterol, a synergistic effect upon the addition of linoleic acid, as well as marked inhibition by lecithin. The capacity of an optimal concentration of linoleic acid and cholesterol (1.5×10^{-5} M) to completely replace the serum requirements of BFU-E was further examined, as shown in Figure 5.5. In contrast to the findings with M-CSF stimulated GM-CFU, only 30% of the maximal growth at 5-10% fetal calf serum could be obtained without fetal calf serum (value at 0% in Fig. 5.5) under these serum free culture conditions, which also fully supported CFU-E growth (data not shown). By morphological criteria, the erythroid bursts detected in these cultures were smaller than those in serum supplemented controls and rather poorly haemoglobinized, as well.

Table 5.6

THE EFFECT OF LIPIDS ON ERYTHROID BURST FORMATION IN SERUM FREE CULTURES

A. Cholesterol and linoleic acid

concentration	BFU-E detected per 10^5 cells: linoleic acid	cholesterol	linoleic + cholesterol
5×10^{-6} M	0	12	16
1×10^{-5} M	0	18	29
1.5×10^{-5} M	0	24	38
2×10^{-5} M	0	24	36

B. Lecithin*

	BFU-E detected per 10^5 cells
linoleic + cholesterol (1.5×10^{-5} M)	28
+ lecithin 4 µg/ml	21
+ lecithin 8 µg/ml	22
+ lecithin 16 µg/ml	15
lecithin alone (4-16 µg/ml)	0

*L-alpha lecithin (β-oleoyl, γ-palmitoyl).
Cultures were stimulated with 15 µl/ml BFA (2.7.5) and 1 unit/ml erythropoietin (2.7.4) added on day 4 of culture. Bursts were scored after 10 days of culture.

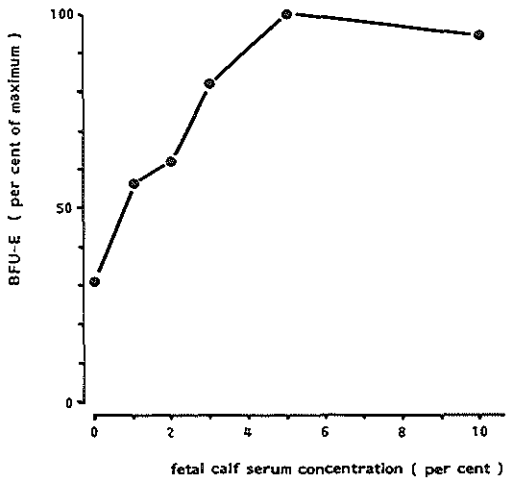


Figure 5.5:
Serum requirements of BFU-E in cultures supplemented with 1.5×10^{-5} M linoleic acid and cholesterol.

Recent reports of the effect of hemin (ferric chloride protoporphyrin IX) on augmentation of haemoglobin synthesis in mammalian reticulocytes (Gross, 1980), as well as on increased detection of erythroid progenitors in hemin-supplemented cultures (Porter et al., 1979; Ibrahim et al., 1982; Monette and Holden, 1982), strongly suggested that this compound may be beneficial to the serum free growth of BFU-E.

Figure 5.6 demonstrates the effect of various concentrations of hemin on the number of BFU-E enumerated 10 days after initiation of serum free cultures. The number of bursts detected increased until a plateau was obtained at 0.1-0.2 mM hemin. At this level, an almost 3-fold higher number of bursts could be enumerated. The addition of hemin to serum free cultures of BFU-E increased not only burst number, but size and red coloured appearance as well. The effect of various hemin concentrations on serum free CFU-E growth is shown in Figure 5.7. The increase in CFU-E plating efficiency, in accordance with previous observations (Porter et al., 1979), can explain the enlargement in BFU-E size by the addition of hemin, since erythroid bursts are composed of numerous clusters.

Figure 5.8 demonstrates the dose response curve for erythropoietin, as detected by the CFU-E assay, in serum free cultures with or without 0.2 mM hemin. No significant alteration in the range of sensitivity to this regulator is induced by hemin. Nonetheless, the hemin-supplemented system seems to show a greater sensitivity for cluster detection at limiting erythropoietin concentrations. This may explain Porter's observations of CFU-E formation in hemin-supplemented cultures devoid of erythropoietin, which, however, contained 30% selected fetal calf serum.

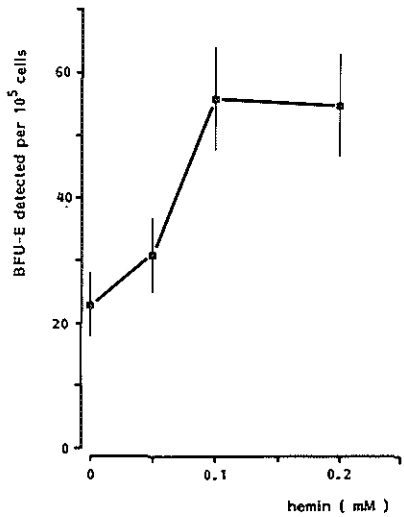


Figure 5.6:
The effect of hemin on mouse marrow erythroid burst (BFU-E) formation in serum free cultures (Legends as in Table 5.5).

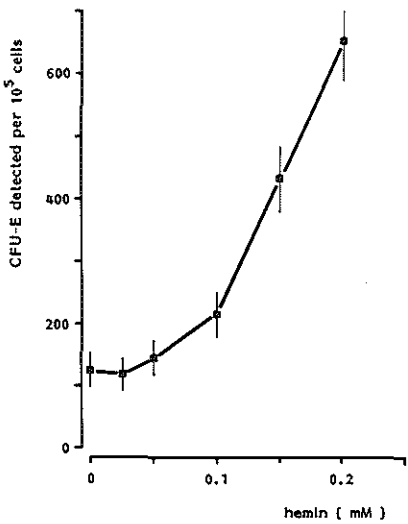


Figure 5.7:
The effect of hemin on mouse marrow erythroid colony (CFU-E) formation in serum free cultures. CFU-E were scored after 3 days of incubation. Cultures were stimulated with a saturating dose of erythropoietin at 0.5 units.ml^{-1}.

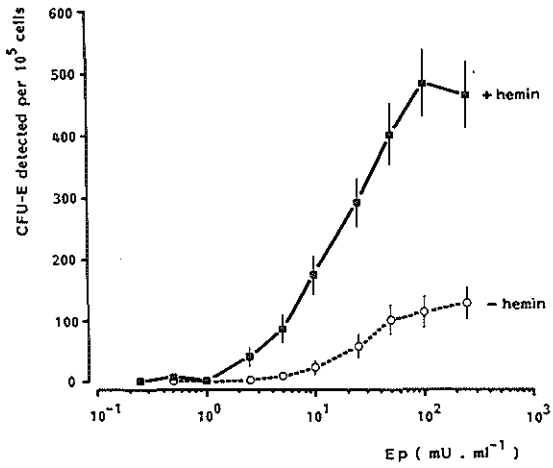


Figure 5.8:
Erythropoietin dose response curve for CFU-E in serum free cultures with or without 0.2 mM hemin.

The mechanism(s) by which hemin enhances the detection of BFU-E in vitro, have not been clarified. Hemin has been suggested to be a primary regulator of the early stages of erythroid differentiation, mimicking the action of BFA (Monette and Holden, 1982). However, the use of selected fetal calf serum as a source of BFA by these investigators, strongly interferes with the distinction between a regulatory or nutritional role of hemin in such cultures, thus raising doubt as to the validity of their conclusions. Such studies can only be performed appropriately under serum free culture conditions.

The presence of BFA is required for the initial stages of erythroid differentiation in vitro. In the absence of BFA, BFU-E rapidly die or lose their capacity to proliferate (Iscove, 1978). The possible BFA-like role of hemin was therefore analysed by its capacity to maintain BFU-E numbers in serum free cultures, to which the addition of BFA was delayed. BFA-supplemented cultures provided with hemin, served as controls. The results of these experiments are shown in Figure 5.9. These findings clearly demonstrate that hemin cannot replace BFA in its capacity to stimulate the proliferation of developmentally early erythroid progenitors. The promotion of burst formation by the delayed addition of hemin to BFA-supplemented cultures may be an artifact due to the disruption of developing bursts by the addition of relatively large volumes (hemin and erythropoietin - 0.15 ml total) in droplets to the culture. Alternatively, hemin may be slightly inhibitory to the initial stages of burst formation.

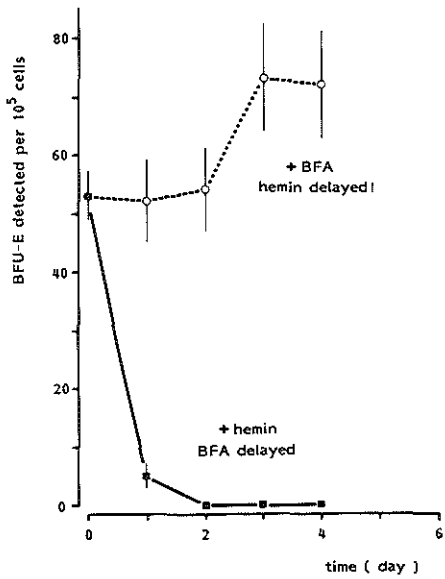


Figure 5.9:
Delayed addition of BFA to hemin-supplemented serum free cultures.

The dose response curve for partially purified BFA in serum free cultures supplemented with hemin, is shown in Figure 5.10. No significant difference in the pattern of responsiveness to BFA due to the presence of hemin can be demonstrated, although the detection of BFU-E at limiting BFA concentrations is slightly more sensitive. This may be related to the effect of hemin on burst size and red-coloured appearance.

Hemin does not replace the requirements of erythroid progenitors for either erythropoietin or BFA (Figures 5.8 and 5.10), neither does it simply replace serum in BFU-E cultures. This is demonstrated in Figure 5.11. The numbers of bursts in hemin supplemented serum free cultures (Figure 5.11A) surpass those obtained maximally at 5-10% fetal calf serum in cultures lacking hemin, by more than 2-fold. Notwithstanding this observation, the plating efficiency in serum free, hemin supplemented cultures is still only 85% of the maximum obtained in serum supplemented controls (Fig. 5.11B). These findings suggest that not all the nutritional requirements provided by serum to cultures of BFU-E, can be replaced by this compound.

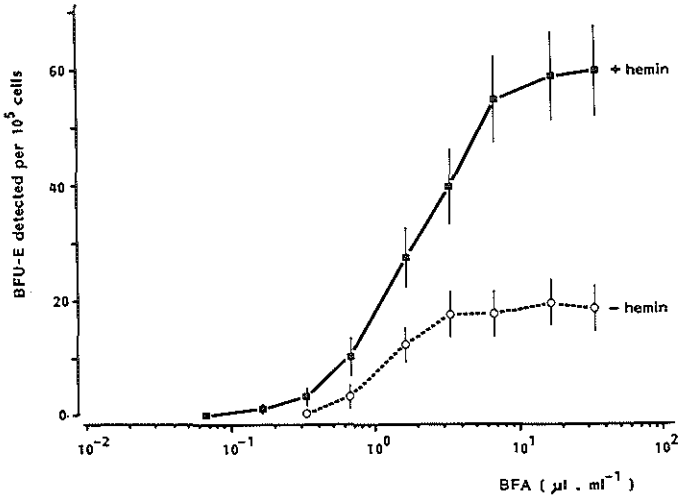


Figure 5.10:
Dose response curve for BFA in serum free cultures with or without 0.2 mM hemin.

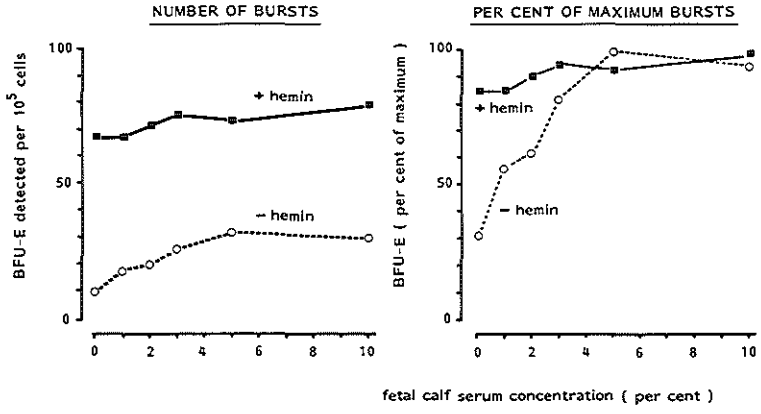


Figure 5.11:
Serum requirements of BFU-E in hemin-supplemented cultures.

5.3 HAEMOPOIETIC STEM CELLS (CFU-S)

Initiation of cell cycling of haemopoietic stem cells *in vitro* has been shown to be triggered by a soluble or humoral factor (Cerny, 1974; Löwenberg and Dicke, 1976, 1977; Wagemaker and Peters, 1978), termed stem cell activating factor, or SAF (Löwenberg and Dicke, 1977). The assay used to detect SAF is based on prolonged maintenance of proliferating CFU-S in suspension cultures. In the absence of this factor, less than 5% of the stem cells, detected by the spleen colony assay, were found to survive for more than three days of culture (Wagemaker and Peters, 1978).

The adverse properties of serum supplemented cultures (batch to batch variability, presence of haemopoietic regulators and/or inhibitors, etc.), apply to the SAF-induced CFU-S maintenance assay, as well. As demonstrated in Table 5.7, replacement of serum in SAF-stimulated cultures with a cocktail consisting of bovine serum albumin, transferrin, selenite, lecithin and 2-mercaptoethanol, enables prolonged maintenance of CFU-S, which is equivalent to that of serum supplemented controls. The recovery of CFU-S in such cultures, is, however, rather low.

Table 5.7

CFU-S RECOVERY IN SAF-STIMULATED SERUM SUPPLEMENTED AND SERUM FREE SUSPENSION CULTURES
(4 days post-initiation)

serum batch (20% FCS)	number of CFU-S harvested (± SD)	per cent recovery (± SD)
1	20 ± 6	28 ± 8
2	22 ± 6	28 ± 8
3	30 ± 7	41 ± 10
none*	16 ± 5	22 ± 7
control-SAF	0	0

3×10^5 marrow cells from 8-12 week-old BCBA F1 female mice were incubated for a period of 4 days in 1 ml suspension cultures stimulated with a saturating concentration of stage II SAF (5 $\mu l.ml^{-1}$). The number of CFU-S of initiation of cultures was 72 ± 7.

*modified Dulbecco's medium supplemented with 0.25% delipidated, deionized BSA, 4×10^{-6} M iron saturated transferrin, 10^{-7} M selenite, 20 $\mu g.ml^{-1}$ egg lecithin and 10^{-4} M mercaptoethanol.

In order to convert the SAF-induced initiation of DNA synthesis not only into prolonged maintenance of CFU-S but also into an actual multiplication of stem cells, a variety of hormones and nutrients was examined for their capacity to further augment the output of stem cells in SAF-stimulated cultures. Hydrocortisone was found to be outstanding in increasing CFU-S numbers over 3-fold. This is shown in Figure 5.12. The effective concentration of hydrocortisone, unable to promote the survival of CFU-S in the absence of SAF, is 10^{-6} M. Dexamethasone and aldosterone had similar effects and were not additive to hydrocortisone. A clear demonstration of the adverse properties of serum in SAF-stimulated cultures is shown in Figure 5.13. Only in one of three serum batches, preselected for optimal growth of haemopoietic cells in vitro, can the enhancement of CFU-S maintenance by hydrocortisone, be expressed. This is another example of the superiority of serum free cultures for the optimal response of haemopoietic cells to stimulation with specific regulators.

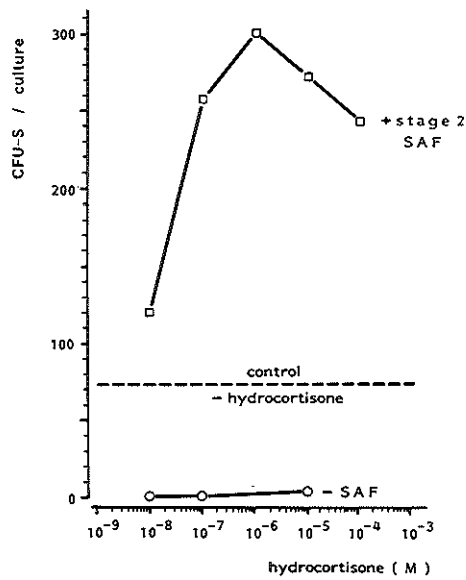


Figure 5.12:
Effect of hydrocortisone on CFU-S in serum free suspension cultures. Culture conditions as described in Table 5.6

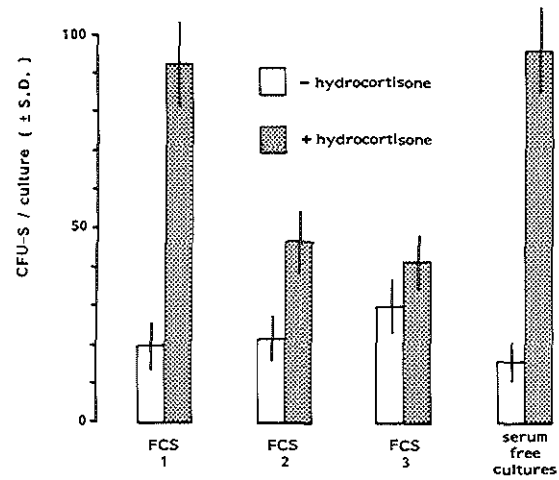


Figure 5.13:
Hydrocortisone in SAF stimulated serum free and serum supplemented cultures.

Serum free, hydrocortisone supplemented SAF cultures yielded a 3-fold recovery of CFU-S within 4 days in culture, as compared to the initial number seeded. This value, being modest in respect to the estimated cell cycle time of 8 hours (Vassort et al., 1973) or the regenerating doubling time of 16 hours to about one day in vivo (Lahiri and Van Putten, 1969; Wagemaker and Visser, 1980), suggested that further attempts to optimize the culture conditions should be pursued. Table 5.8 lists the substances titrated over a wide range of concentrations for their effect on CFU-S numbers in hydrocortisone supplemented serum free cultures, both in the presence and the absence of SAF. Among the hormones tested, testosterone has a slight additive effect, whereas the beta specific cathecholamines isoproterenol ($\beta_1\beta_2$) and albuterol (β_2), promoted SAF-induced multiplication of CFU-S to an over 4-fold increase in recovery after 4 days in culture. These findings are demonstrated in Figure 5.14. β-specific cathecholamines as well as testosterone were demonstrated by Byron (1972; 1972a) to stimulate cell cycle activation of CFU-S. Neither of these, however, nor any of the other substances tested, were capable of promoting survival of CFU-S in the absence of SAF. Furthermore, whereas a β_2 specificity for the enhancement of SAF-stimulated multiplication of CFU-S in serum free cultures has been observed, a β_1 specific effect was reported by Byron.

Among the components present in the serum free supplemented mixture, the requirement for albumin seems to be most crucial, although its optimal concentration is 4-fold lower than the one required in viscuous cultures, where it may be needed to buffer inhibitory substances present in methyl-

Table 5.8

THE EFFECT OF VARIOUS COMPOUNDS ON CFU-S RECOVERY IN HYDROCORTISONE SUPPLEMENTED SERUM FREE SUSPENSION CULTURES

hormones	+SAF	-SAF
testosterone (10^{-10}–10^{-5} M)	±	
oestradiol (10^{-9}–10^{-4} M)	-	n.d.
progesterone (10^{-9}–10^{-4} M)	-*	n.d.
thyroid hormones T3, T4 (0.001–1 mg.l^{-1})	-	-
insulin (0.01–100 mg.l^{-1})	±	-
glucagon (10^{-6}–10^{-9} M)	-	n.d.
phenylephrine (α) (10^{-8}–10^{-4} M)	-	n.d.
norepinephrine ($\alpha\beta_1$) (10^{-9}–10^{-4} M)	-	n.d.
isoproterenol ($\beta_1\beta_2$) (10^{-9}–10^{-5} M)	+	-
albuterol (β_2) (10^{-8}–10^{-4} M)	+	-
nutrients		
albumin (2.5 g.l^{-1})	++	-
transferrin.Fe_2 (4 x 10^{-6} M)	±	-
Na_2SeO_3 (10^{-7} M)	±	-
2-mercaptoethanol (10^{-4} M)	±	-
nucleosides (10^{-3} g.l^{-1})	+	-
lipoic acid (10^{-5}–10^{-9} M)	-	-
glutathione (10^{-3}–10^{-7} M)	±	-
ceruloplasmin (0.01–30 mg.l^{-1})	-	-

++: increase over 2-fold compared to control
+ : increase between 1.5 and 2-fold
± : increase less than 1.4-fold
- : no effect
-*: inhibition

cellulose. Among several new components tested, the addition of a mixture of nucleosides to SAF-stimulated cultures, has further improved CFU-S output. A further point of interest is the effect of lipids on CFU-S recovery. This is shown in Table 5.9. A substantial amount of stem cells is maintained in the absence of exogenously added lipids. Furthermore, an increased amount of stem cells is recovered in the presence of either lecithin or the linoleic acid-cholesterol mixture, the former found to be inhibitory to the growth of granulocyte-macrophage and erythroid progenitors.

Stem cells are functionally defined by their capacity to provide protection from the fatal consequences of bone marrow failure (Trentin and Fahlberg, 1963). In order to evaluate whether SAF-stimulated CFU-S in serum free cultures meet this criterion, the thirty day radioprotective capacity of cells from 4-day suspension cultures was examined. The results of this study shown in Figure 5.15, demonstrate that stem cells obtained from SAF-stimulated serum free suspension cultures are equally effective as fresh marrow stem cells, at equivalent CFU-S numbers, in providing protection from lethal irradiation.

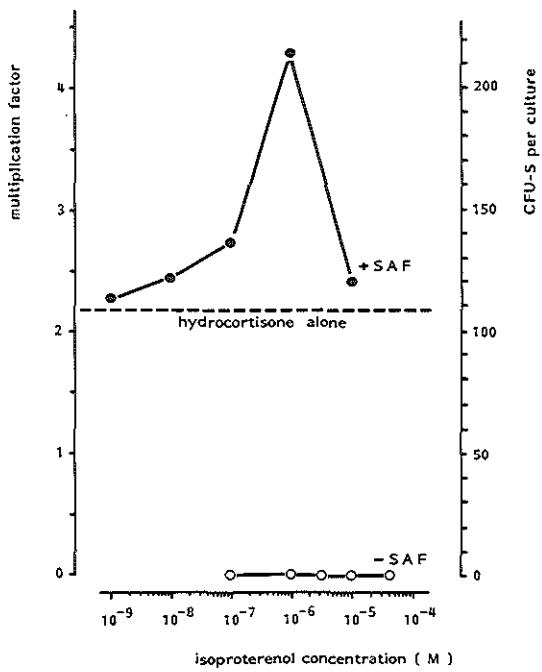


Figure 5.14:
The effect of isoproterenol ($\beta_1\beta_2$) on CFU-S recovered in SAF-stimulated suspension cultures.

Table 5.9

THE EFFECT OF LIPIDS ON CFU-S RECOVERY IN SAF-STIMULATED SERUM-FREE SUSPENSION CULTURES

	CFU-S recovered per culture ± S.D.
lecithin (20 µg/ml)	122 ± 12
linoleic acid + cholesterol (1×10^{-5} M)	115 ± 12
control - lipid	77 ± 8

The CFU-S concentration at the initiation of culture was 54 ± 7

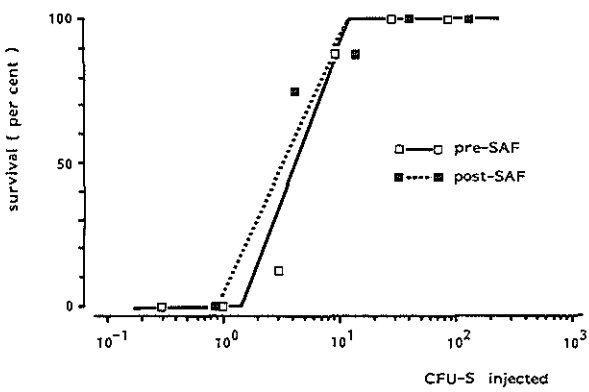


Figure 5.15:
Survival of lethally irradiated mice injected with fresh or cultured bone marrow cells. Lethally irradiated (8.75 Gy) groups (n = 15) of BCBA F1 female mice were transplanted with increasing numbers of freshly explanted or SAF-cultured (4 days) marrow cells. The CFU-S concentration was determined in separate groups of mice (n = 8), transplanted with appropriate dilutions of the same cell suspensions, and killed 8 days thereafter.

5.4 CONCLUSIONS

The proliferation and maturation of haemopoietic cells in vitro requires stimulation with specific haemopoietic factors such as erythropoietin for erythroid cells, several types of colony stimulating factors for progenitors of granulocytes and macrophages, etc. In this chapter, the response of haemopoietic stem cells and progenitor cells to stimulation with these factors in defined, serum free cultures, is described. Whereas much effort has been placed on the purification of haemopoietic regulators and characterization of the specific target cell populations which they stimulate (Sheridan and Metcalf, 1973; Bol et al., 1979; Nicola et al., 1981; Stanley and Heard, 1977; Burgess et al., 1977), the data presented here stress the critical role of the basic nutritional and/or hormonal growth requirements of haemopoietic cells in determining their capacity to respond to these factors.

The development of serum free cultures for the growth of a large variety of mammalian cells, has been based on the replacement of serum with the appropriate nutrients and/or hormones which it contains and which each cell type individually requires for optimal growth (reviewed by Barnes and Sato, 1980). Several basic nutritional requirements seem to be shared by haemopoietic cells which differ in both lineage and developmental stage. This is demonstrated, for example, by the effect of various lipids on the serum free growth of GM-CFU and BFU-E. The inhibition of colony and burst formation by lecithin is in accordance with the essential need for cholesterol by such cells; phospholipid has been shown to critically influence the uptake of cholesterol into erythrocyte membranes (Cooper et al., 1975). Although the multiplication of stem cells in SAF-stimulated serum free suspension cultures differs in its lipid requirements, these results are difficult to interpret, since the extent of stem cell doubling in such cultures might also be related to conditions influencing cellular differentiation. Accurate interpretation as to the role of various nutrients and hormones in stem cell multiplication in vitro, must therefore await the use of highly purified SAF preparations and/or stem cells, in such cultures.

Although lipids are important for the growth of haemopoietic cells, their presence in culture does not always completely eliminate the requirement for serum. For example, the BFA-stimulated proliferation of developmentally early erythroid progenitors requires additional components, whose role, however, diminishes as these cells mature. In this as well as other respects, hemin, although capable of promoting an extensive degree of BFU-E growth in marrow cultures devoid of serum, is not a true serum substitute. Nevertheless, under certain pathological conditions in which high concentrations of this component may circulate (i.e., extensive haemolysis), hemin may indeed play a role in enhancing erythropoiesis. The nature of the additional serum-replacing requirements for BFA-induced BFU-E proliferation is not yet known. The effect of a variety of serum components (i.e., hormones, vitamins, etc.) on such cultures, has yet to be investigated.

Not all serum-replacing components act nonspecifically on haemopoietic cells. This is exemplified by the hydrocortisone requirement for complete proliferation and maturation of GM-CSF stimulated colony formation by GM-CFU in serum free cultures, while slightly inhibiting the response of

these cells to M-CSF. A specific requirement of hydrocortisone for granulocyte formation has been implied by the recent observations of Kubota et al. (1983), in which murine marrow GM-CFU were found to produce a highly reduced proportion of granulocytic colonies in agar cultures devoid of serum, which fully supported macrophage colony formation. Conclusive evidence for a modulatory effect of hydrocortisone on the direction of differentiation of bipotential granulocyte-macrophage progenitors, awaits a more detailed time-course study, in which enriched populations of such cells are stimulated with purified GM-CSF in serum free cultures which contain or lack this hormone.

Although serum provides a large variety of components to cultures of haemopoietic cells, these are not, however, necessarily suitable for the optimal response of every cell type to its specific regulator. For example, the formation of macrophage colonies in serum free cultures is superior to that of serum supplemented controls. The possible inhibition of cholesterol uptake by serum (Table 5.3), or the presence of hormones which may inhibit macrophage colony formation (Figure 5.4), are only two of numerous possible explanations. The effect of hydrocortisone on the enhancement of SAF-induced stem cell doubling, is often masked by serum (Figure 5.13), as well. The availability of serum free cultures for the growth of murine haemopoietic cells not only enables the controlled optimization of culture conditions to a greater extent, but, of greater relevance to this field of research, accurate studies of the mechanisms involved in the regulation of haemopoietic cell differentiation and its disruption in disease, can now be performed.

CHAPTER VI

SENSITIVITY OF STEM CELLS AND PROGENITOR CELLS FROM RLV-INFECTED MICE TO STIMULATION WITH HAEMOPOIETIC REGULATORS IN SERUM-FREE CULTURES

The data presented in this chapter deal with studies concerning the effect of Rauscher infection on the responsiveness of haemopoietic cells to specific factors which regulate their proliferation and maturation in vitro. Since infection of W/W^V mutants with RLV has provided direct evidence for the involvement of the haemopoietic stem cell in Rauscher disease, evaluation of the responsiveness to those regulators which act upon stem cells or their immediate descendants, is of major relevance to these studies. Although the effect of RLV on regulator sensitivity of haemopoietic cells has been previously investigated, these studies, limited only to the erythroid compartment, have provided conflicting results (Hasthorpe and Bol, 1979; Gallicchio and Murphy, 1981; Walma and Wagemaker, 1979; Ostertag and Pragnell, 1981). The importance of serum free cultures for the accuracy of such analyses has already been mentioned (Chapter 5).

Defective macroscopic spleen colony formation by stem cells of W/W^V mice reflects a reduced capacity for haemopoietic cell differentiation. Infection of these mice with Rauscher Virus restores their macroscopic spleen colony forming capacity. Analysis of the effect of both the W mutation and RLV infection on the sensitivity of haemopoietic cells to specific regulator molecules may, in addition, provide an explanation to the mechanism(s) by which Rauscher Virus repairs the W/W^V stem cell differentiation defect.

6.1 THE EFFECT OF RLV INFECTION ON THE RESPONSIVENESS OF HAEMOPOIETIC CELLS TO STIMULATION WITH SPECIFIC REGULATORS IN VITRO

The dose response curves for haemopoietic factors stimulating stem cell replication (SAF) and the developmentally early stages related to differentiation of the erythroid and granulocyte-macrophage pathways (BFA and CEA, respectively), are given in Figures 6.1-6.3. These findings fail to demonstrate any significant effect of Rauscher infection on the in vitro responsiveness (i.e., threshold concentration, range of sensitivity, saturation dose) of either the haemopoietic stem cell or its immediate descendants, defined by specific assays in vivo and in vitro, to these regulators. This is observed for progenitors of both splenic and femoral origin, wherever tested. In all cases it is obvious that infection with RLV resulted only in the spleen in an increased number of progenitor cells.

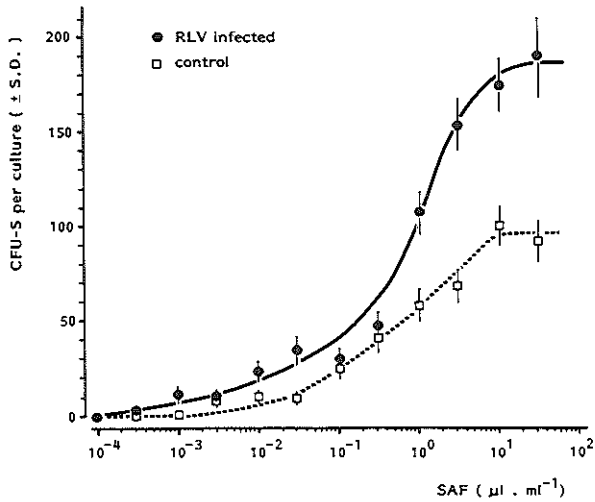


Figure 6.1:
Dose response curve for SAF in serum free suspension cultures of marrow CFU-S from RLV-infected mice.
Stage III SAF (2.7.5) was used for these assays. Cultures consisted of 3 x 10^5 marrow cells per ml. Cell donors were BALB/c females aged 8 weeks, infected one week previously with 0.4 ml RLV cell free extract, or control uninfected mice of similar age and sex. Cultures were terminated 4 days after initiation. Lethally irradiated (8.75 Gy γ-rays) BALB/c mice were used as recipients. The initial number of CFU-S per ml for RLV-infected and control cultures was 95 ± 8 and 43 ± 6, respectively.

The similar enrichment of CFU-S in suspension cultures of RLV-infected and control BALB/c marrow cells, stimulated with a saturating dose of SAF (2.3-fold versus 2.1-fold, respectively), demonstrates that the extent of stem cell doubling in vitro is unaltered as a result of RLV infection, as well. The BFA dose response data interestingly contrast with previous reports of increased BFA sensitivity found in splenic BFU-E only (>1000-fold as compared to normal; Walma and Wagemaker, 1979), or both in spleen and marrow BFU-E (Ostertag and Pragnell, 1981) of RLV-infected mice. The former observation suggests that this property may not be exclusively determined by an inherent alteration in the progenitor cells themselves.

In terms of erythropoietin requirement for burst formation in serum free cultures, this is unaltered by Rauscher infection as well, as shown in Figure 6.4. Previous claims of increased sensitivity of RLV- infected BFU-E to erythropoietin (Hasthorpe and Bol, 1979), are not justified by the data shown by these authors, since the presentation (and interpretation) of normalized titration curves is only valid under conditions in which complete saturation has been obtained. The findings presented here, as well as the unaltered

requirement for erythropoietin in cultures of late erythroid progenitors (CFU-E) from RLV-infected mice, demonstrated by several investigators (Opitz et al., 1977; Hasthorpe, 1978; Gallicchio and Murphy, 1981), are well in accordance with the suppressive effect of hypertransfusion on RLV-induced erythroblastosis (Dunn et al., 1966).

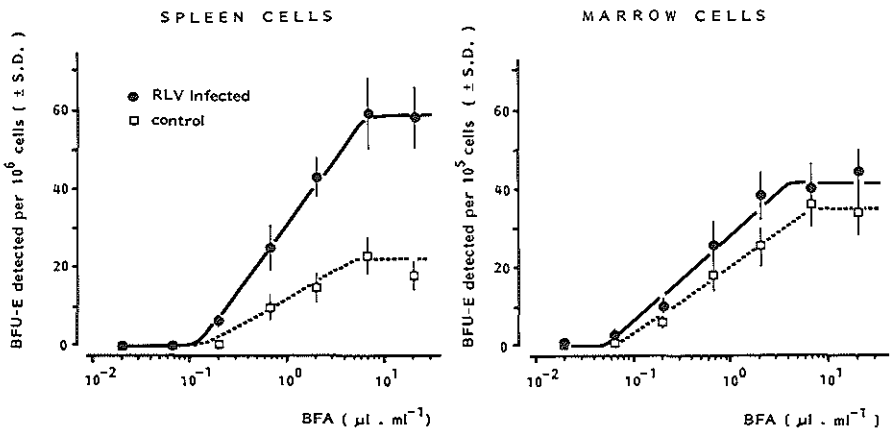


Figure 6.2:
BFA dose response curve in serum free cultures of spleen and marrow BFU-E from RLV-infected mice.
One week post RLV-infection of BD_2 +/+ mice. Uninfected +/+ littermates were used as controls. The source of BFA was a stage II preparation of medium conditioned by Con-A stimulated mouse spleen cells (2.7.5). The addition of erythropoietin (1 $lu.ml^{-1}$) was delayed until day 4 of culture. Bursts were scored at day 10 of culture. Results represent a mean of three independent experiments.

Although leukocytosis is a common feature of Rauscher disease, in vitro investigations into the regulation of granulocyte-macrophage progeny production have been uninvestigated, probably due to the fact that the main interest in Rauscher disease, due to its most prominent features, lies within the erythroid compartment. Nevertheless, the responsiveness of GM-CFU from RLV-infected BD_2 mice, to stimulation with two purified colony stimulating factors, M-CSF and GM-CSF, was examined in serum free cultures. The results are shown in Figures 6.5 and 6.6. As with all other previous assays, no difference in the dose response patterns obtained with GM-CFU from RLV-infected or control mice, can be demonstrated. Although a plateau was not observed in GM-CSF stimulated cultures, these are, nevertheless, identical in threshold concentrations and slopes.

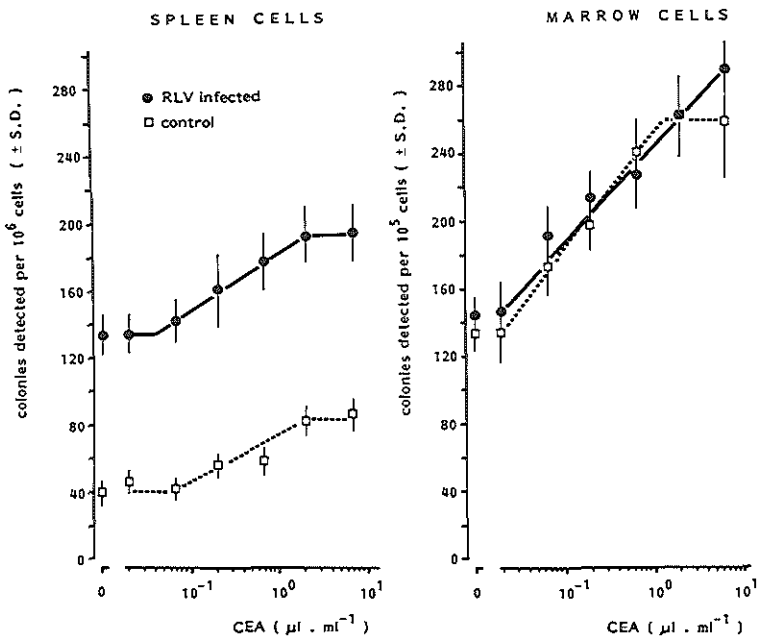


Figure 6.3:
Dose response curve for colony enhancing activity (CEA) in serum free cultures of spleen and marrow GM-CFU from RLV-infected mice.
1 week post RLV-infection of BD_2 +/+ mice. Uninfected +/+ littermates were used as controls. The source of CEA was a stage II preparation of MSCM (2.7.5). This preparation did not have any detectable colony stimulating activity at the highest concentration used, when added alone. M-CSF (2.7.3) was added at a saturating concentration of 4.7 µg.ml^{-1}. Colonies were scored 7 days after initiation of culture.

In view of the findings observed with all regulators examined, a test for the capacity of the specifically designed serum free cultures to detect "hormone-independent" haemopoietic cell proliferation and maturation, was performed. An example of such is the erythropoietin-independent formation of erythroid clusters in vitro, by erythroid precursors from mice infected with the polycythemic strain of Friend virus (FLV-P) (Horoszewicz et al., 1975; Liao and Axelrad, 1975; Rossi and Peschle, 1980; MacDonald et al., 1980a). Marrow and spleen cells from FLV-P infected BALB/c mice were assayed for CFU-E in serum free cultures with and without erythropoietin. CFU-E cultures from RLV infected or uninfected mice were used as controls. The results are shown in Table 6.1. These findings show that the lack of altered responsiveness to haemopoietic regulators as a result of RLV infection is not due to a deficiency in capacity of the serum free culture system to detect growth factor independence.

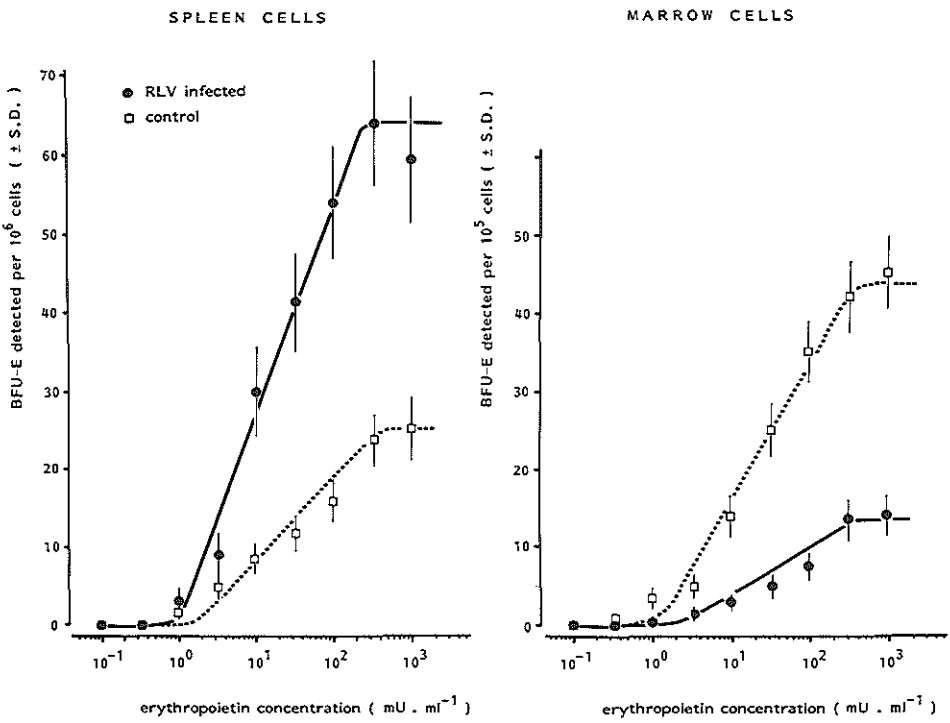


Figure 6.4:
Erythropoietin dose response curve in serum free cultures of BFU-E from RLV-infected mice.
One week post RLV-infection of BD_2 +/+ mice. Uninfected littermates were used as controls. BFA was added at a saturating concentration of 10 $\mu l.ml^{-1}$. Colonies were scored 10 days after initiation of culture.

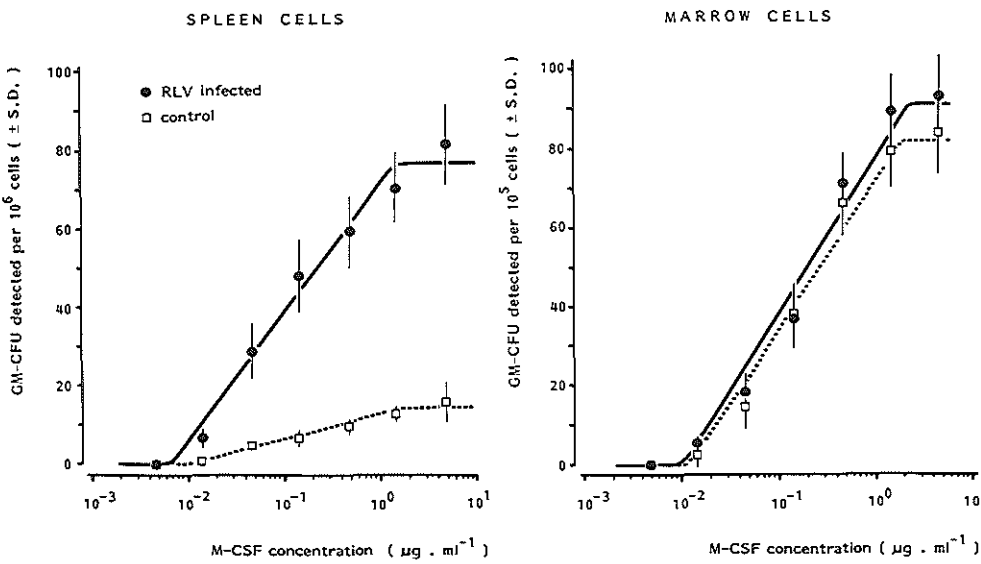


Figure 6.5:
M-CSF dose response curve in serum free cultures of GM-CFU from RLV-infected mice. One week post RLV-infection of BD_2 +/+ mice. Uninfected +/+ littermates were used as controls. Colonies were scored 7 days after initiation of culture.

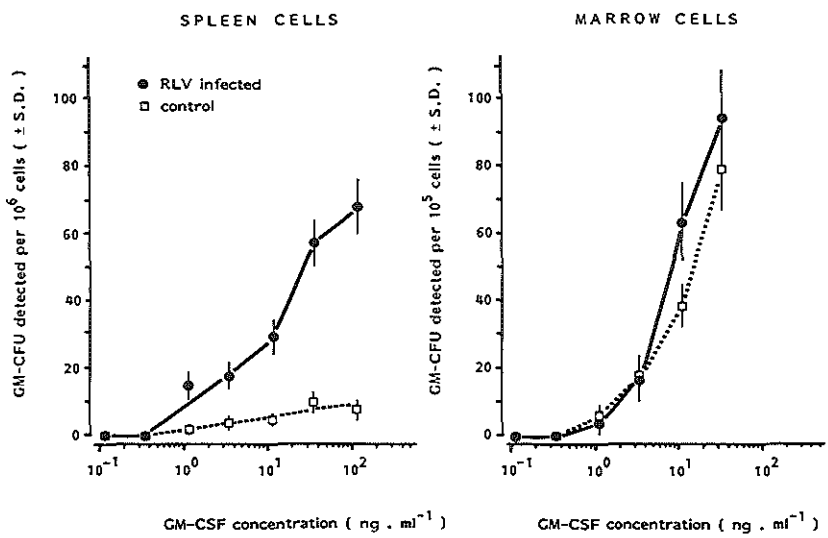


Figure 6.6:
GM-CSF dose response curve in serum free cultures of GM-CFU from RLV-infected mice. One week post RLV-infection of BD_2+/+ mice. Uninfected +/+ littermates were used as controls. Colonies were scored 7 days after initiation of culture.

Table 6.1

ERYTHROPOIETIN-INDEPENDENT CFU-E GROWTH
IN SERUM FREE CULTURES

virus* injected	spleen CFU-E/10^6 cells ± SD -EP	spleen CFU-E/10^6 cells ± SD +EP**	femur CFU-E/10^5 cells ± SD -EP	femur CFU-E/10^5 cells ± SD +EP**
FLV-P	13,400 ± 1637	17,000 ± 1843	1105 ± 121	1662 ± 144
RLV	251 ± 79	11,254 ± 1503	0	138 ± 34
uninfected control	0	201 ± 50	0	631 ± 89

*BALB/c females, aged 5-6 weeks, one week post-injection of 0.4 ml cell free extract.
**EP was added at a concentration of 0.5 unit.ml^{-1}.

6.2 RESPONSIVENESS OF HAEMOPOIETIC CELLS FROM RLV INFECTED W/W^V MICE TO STIMULATION WITH SPECIFIC REGULATORS IN VITRO

Defective macroscopic spleen colony formation by stem cells of W/W^V mutants reflects a reduced capacity for haemopoietic cell differentiation. The means by which the W mutation exerts this effect is far from clear. Apart from claims of unaltered erythropoietin sensitivity of erythroid progenitors assayed in vitro (Gregory and Eaves, 1978; Wagemaker and Visser, 1979; Iscove, 1980), no other information concerning the effect of the W gene on the responsiveness of haemopoietic cells to stimulatory influences, those regulating the early stages of erythroid differentiation being of major relevance, is available.

Infection of W/W^V mice with Rauscher virus restores their spleen colony forming capacity. Although the data presented so far fail to demonstrate any alteration in the sensitivity of the HSC or progenitors to stimulation with haemopoietic regulators as a result of Rauscher infection, the possibility that spleen colony formation by RLV-infected W/W^V stem cells reflects a correction of impaired responsiveness to stimulation, resulting from the W mutation, justifies these investigations.

Due to the lack of a spleen colony assay for W/W^V stem cells, as well as the highly reduced CFU-S frequency in RLV-infected W/W^V marrow cells (3.2.2), a comparative analysis of W/W^V stem cell replication in vitro induced by SAF, was not feasible. The responsiveness of erythroid (BFU-E) and granulocyte-macrophage (GM-CFU) progenitors from W/W^V mutants to stimulation in vitro with haemopoietic regulators, examined in relation to their genotype and to RLV infection, is shown in Figures 6.7-6.10. No effect of

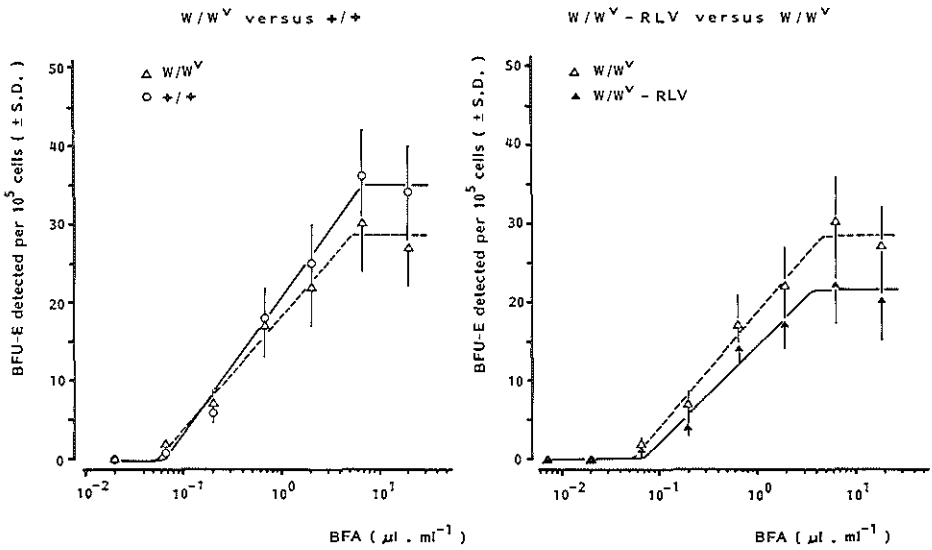


Figure 6.7:
BFA dose response curve in serum free cultures of BFU-E from W/W^V mice. Bone marrow cells from BD_2F1 littermates of W/W^V and +/+ genotypes were used for these experiments. B. One week post RLV-infection.

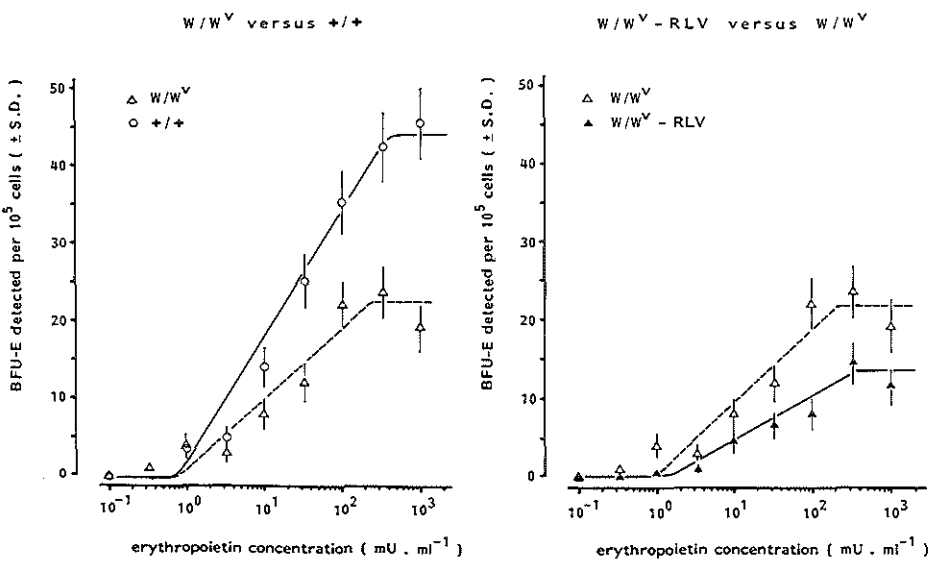


Figure 6.8:
Erythropoietin dose response curve in serum free cultures of BFU-E from W/W^V mice. For legends, see figure 6.7.

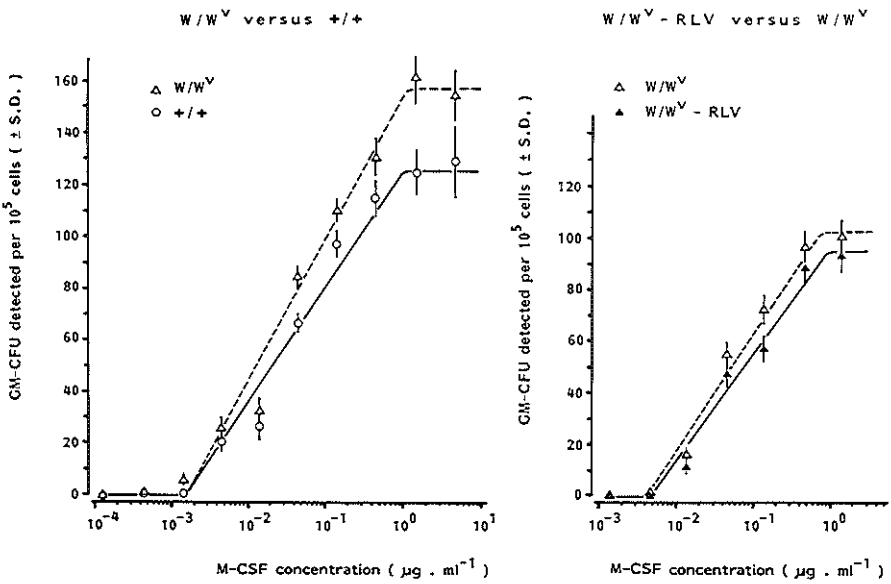


Figure 6.9:
M-CSF dose response curve in serum free cultures of GM-CFU from W/W^V mice. For legends, see figure 6.7.

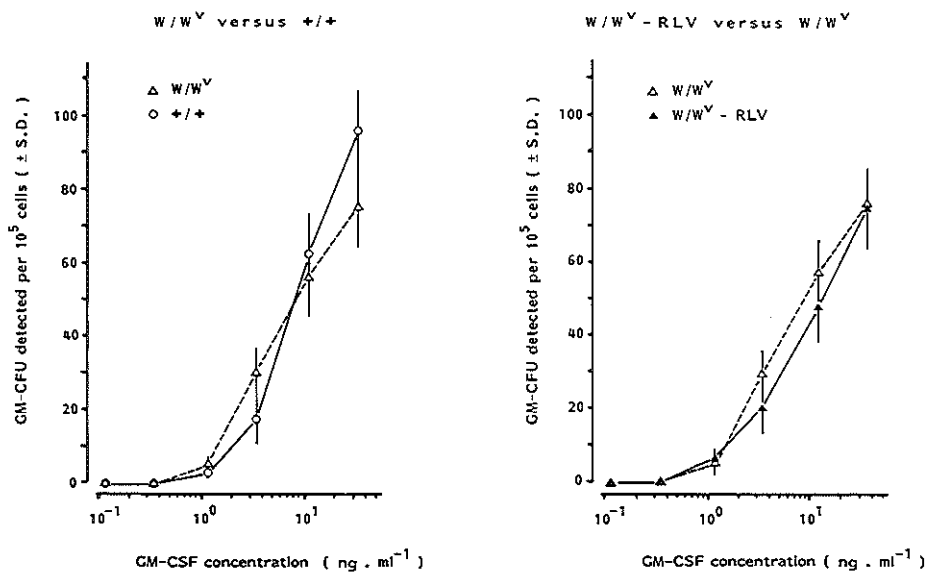


Figure 6.10:
GM-CSF dose response curves in serum free cultures of GM-CFU from W/W^V mice. For legends, see figure 6.7.

the W mutation on the sensitivity to stimulation with any of the regulators titrated (6.7-6.10A), can be demonstrated. Rauscher infection of W/W^V mice did not alter the pattern of responsiveness to these regulators (6.7-6.10B), either. Colony and burst size remained the same regardless of genotype or RLV infection.

6.3 CONCLUSIONS

The findings presented in this chapter fail to demonstrate any effect of Rauscher infection on the sensitivity of haemopoietic cells from either normal or mutant W/W^V mice, to specific factors which stimulate their proliferation and maturation in vitro. Of special interest in these observations, obtained in serum free cultures specifically adapted for the optimal detection of such cells in vitro, is the contrast with previous observations related to BFA-independent BFU-E growth from RLV-infected mice (Walma and Wagemaker, 1979; Ostertag and Pragnell, 1981). A possible explanation for this discrepancy may lie in the use of defined culture conditions, which, having been specifically designed for the detection of haemopoietic cells, may not necessarily be suitable for the expression of those cells that produce and/or release increased amounts of BFA into the culture system.

Evidence for the effect of retroviral infection on the enhancement of environmental stimulatory influences has been provided by several recent observations, such as the nonlinearity of cell to BFU-E regression in BFA-devoid cultures from Friend Leukemia Virus (FLV)-infected mice (Peschle et al., 1983), the presence of BFA in lectin-free medium conditioned by spleen cells from FLV-infected mice (idem), as well as the enhancement of GM-CSF production by several fibroblast cell lines following infection with various murine leukemia retroviruses (Koury and Pragnell, 1982). These recent observations also point out the risk in interpretation of regulator-sensitivity studies, in which a heterogeneous population of cells that respond to factors, as well those producing them (Wagemaker, 1978; 1980), may be present in the culture system.

The findings presented in this chapter further demonstrate that the W/W^V stem cell defect cannot be attributed to any abnormality in the responsiveness of W/W^V haemopoietic cells to stimulation with known haemopoietic regulators (Figures 6.7A-6.10A). Unfortunately, it was not feasible to the SAF response of RLV-infected W/W^V CFU-S. Evidence that enhanced factor production is not the primary mechanism by which Rauscher virus restores the W/W^V stem cell differentiation defect, has also been presented (Chapter 3, Table 3.4). The in vitro studies described in this chapter thus do not explain the lesions exerted by the W mutation or the effect of RLV infection, on stem cell expression. In an attempt to further elucidate these questions, additional studies were performed in vivo. These are described in the following chapter.

CHAPTER VII

DIFFERENTIATION AND SELF REPLICATION OF STEM CELLS IN RELATION TO THE W MUTATION AND RLV INFECTION

Two major features of haemopoietic stem cells, defined as such, are pluripotentiality and extensive self renewal. Due to the lack of a suitable assay for stem cell enumeration in the W/W^V mouse, comparative studies related to the role of the W gene in the expression of both these properties have been difficult to perform, as well as to interpret. Infection of W/W^V mice with Rauscher Virus has restored their macroscopic spleen colony forming capacity, thus enabling enumeration of functional stem cells by the spleen colony assay. Evaluation of the role of the W gene in stem cell differentiation and self renewal under controlled experimental conditions, can now be performed. Analysis of the effect of RLV infection on these properties, may contribute to the understanding of the mechanism by which RLV exerts its influence upon W/W^V stem cell expression.

7.1 DIFFERENTIATION OF W/W^V STEM CELLS

Analysis of the generation of haemopoietic progeny in spleens and femurs of lethally irradiated hosts transplanted with haemopoietic cells, is the only direct assay presently available for studies of stem cell differentiation. Evaluation of the lesion exerted by the W mutation upon this property, has been performed by comparisons based on grafting equivalent numbers of W/W^V and +/+ marrow cells, whose relative stem cell content could not, however, be determined (Sutherland et al., 1970; Harrison, 1972; Wagemaker and Visser, 1979). Restoration of the spleen colony forming capacity of W/W^V stem cells by Rauscher infection, thus enabling their direct enumeration, has now made controlled investigations in which known numbers of +/+ and W/W^V marrow stem cells are present in the grafted cell suspensions, feasible. The results of comparative analysis of progeny production by W/W^V and +/+ stem cells, whose number in the transplanted cell suspensions approaches similar values, are shown in Table 7.1. The comparison is based on the fact that, within certain ranges, RLV infection does not influence CFU-S frequency, which is assumed to apply also to W/W^V stem cells.

A highly reduced production of both granulocyte-macrophage, as well as erythroid progenitors, is observed in the spleen of mice receiving the W/W^V haemopoietic cell graft. In contrast to the observations in the spleen, the number of progenitors generated in the femur by W/W^V stem cells is normal, even slightly higher than those generated by +/+ stem cells. This may result from the detection of progenitor cells, due to the 100-fold higher number of W/W^V marrow cells injected.

Table 7.1

GENERATION OF HAEMOPOIETIC PROGENITORS BY W/W^V STEM CELLS
(8 days post-transplantation)

marrow cell number injected		stem cells** injected (CFC-S)	progenitors per spleen ($\times 10^3$) ± SD			progenitors per femur ($\times 10^3$) ± SD		
			CFU-E	GM-CFU	BFU-E	CFU-E	GM-CFU	BFU-E
1.5×10^5	+/+*	2322 ± 790	484 ±43	35 ±0.4	1.8±0.2	2.0±0.15	0.8±0.06	0.01±0.004
1.5×10^7	W/W^V	1600-2500	7.3±0.78	0.6±0.09	0.4±0.07	3.5±0.69	1.7±0.26	0.22±0.3

* BD_2 +/+ littermates of similar age and sex.

**Calculated on the basis of spleen colony counts and the seeding fraction f (Table 3.7).

The estimated number of CFU-S in W/W^V marrow is based on spleen colony formation by RLV-infected W/W^V stem cells (Table 3.5) on the assumption that RLV-infection does not influence stem cell frequency in W/W^V mice.

Lethally irradiated (9.0 Gy gamma rays) BD_2 W/+ or W^V/+ mice were used as recipients.

Femurs or spleens from three mice were pooled.

Previous observations of defective W/W^V progeny production in the femur (Sutherland et al., 1970; Wagemaker and Visser, 1979), are undoubtedly due to the fact that more stem cells were present in the +/+ marrow graft.

7.2 SELF REPLICATION OF W/W^V STEM CELLS

The formation of histologically detectable microcolonies upon retransplantation of spleens from irradiated mice transplanted with W/W^V marrow, has indicated that these colonies originate from self renewing, haemopoietic stem cells (Lewis et al., 1967). However, due to the difficulties encountered in accurate enumeration of stem cells in such spleens, however, studies of the role of the W gene in stem cell self renewal were not further pursued. The capacity of RLV-infected W/W^V stem cells to form macroscopic spleen colonies, has enabled evaluation of this property by the spleen colony assay.

Spleen cell suspensions obtained from either BD_2-W/W^V or +/+ littermates infected 21 days previously with Rauscher virus, or from control uninfected +/+ mice, were used as a source of stem cells. These were transplanted into lethally irradiated recipients, whose spleens served as a source of stem cells for a subsequent transplantation, performed 10 days later (described in 2.4.1). The results of this study are shown in Table 7.2. The values obtained demonstrate an approximately 10-fold reduction in the capacity of RLV-infected W/W^V CFU-S to produce cells that give rise to secondary spleen colonies upon retransplantation. Since RLV infection does not seem to modify the self renewal capacity of +/+ stem cells in any direction, as already indicated by the similar extent of stem cell enrichment in SAF-stimulated suspension cultures (6.1), the diminished self replication of RLV-infected W/W^V stem cells has to be attributed to the W/W^V genome. Defective W/W^V stem cell self renewal is additionally demonstrated by the CFU-S frequency observed in spleens and femurs of W/W^V recipients which had been lethally irradiated and reconstituted 150 days previously with RLV-infected W/W^V stem cells. These findings, shown in Table 7.3, further demonstrate that this defect, in contrast to stem cell differentiation, is expressed not only in the spleen, but in the femur as well.

7.3 GENERATION OF HAEMOPOIETIC PROGENITORS BY RLV-INFECTED STEM CELLS

The effect of RLV infection on the generation of haemopoietic progenitors by W/W^V stem cells is demonstrated in Table 7.4. An increase in production of all progenitors assayed in the spleen, is clearly evident. This demonstrates that the repair of W/W^V stem cell differentiation by RLV infection is not limited to the erythropoietic pathway, as might have been suggested from the prominent erythroid changes following RLV infection and from the fact that the macroscopic visibility of the majority of spleen colonies is due to erythroid cells (Bleiberg et al., 1965; Curry and Trentin, 1967). A remarkable observation in these data is that production of progeny in the femur by W/W^V stem cells, is unaltered by RLV infection.

Table 7.2

FORMATION OF SECONDARY SPLEEN COLONIES BY CFU-S FROM EITHER RLV-INFECTED W/W^V, +/+ OR UNINFECTED +/+ DONORS

genotype donor	primary graft cell number grafted	number of spleen colonies ± SD	secondary graft, number of spleen colonies detected per primary spleen** ± S.D.	number of secondary spleen colonies per primary spleen colony
W/W^V RLV*	2×10^7	11.8 ± 1.4	11.6 ± 1.5	1.2 ± 0.2
	5×10^7	29.5 ± 3.5	40.4 ± 3.7	
+/+ RLV*	5×10^5	10.4 ± 0.9	129.2 ± 9.0	12.4 ± 1.2
+/+	5×10^5	7.9 ± 0.8	73.9 ± 4.9	9.4 ± 1.1

* 3 weeks post-RLV-infection.
**Determined by injecting cell suspensions from primary recipient spleens, at various dilutions, into secondary irradiated recipients.

Lethally irradiated (9.0 Gy) BD_2-W^+ or W^V/+ mice were used as recipients.

Table 7.3

CFU-S FREQUENCY IN LONG TERM SURVIVING BD_2-W/W^V MICE RECONSTITUTED WITH RLV-INFECTED W/W^V SPLEEN CELLS (150 days post transplantation)

CFU-S frequency	W/W^V RLV*	+/+**	+/+ RLV**	+/+*** controls
spleen (per 10^7 cells)	3.2 ± 0.6	n.d.	121 ± 14	136 ± 12
femur (per 10^6 cells)	9.9 ± 1.0	156 ± 26	170 ± 25	243 ± 23

* 2×10^7 spleen or 2×10^6 marrow cells from long-term surviving W/W^V mice were injected into lethally irradiated (9.0 Gy) BD_2-W/+ or W^V/+ littermates.
** 5×10^5 spleen or 5×10^4 marrow cells from long-term surviving W/W^V mice transplanted with +/+ or +/+ RLV cells.
*** BD_2 +/+ littermates, 8-12 weeks old.

Table 7.4

GENERATION OF HAEMOPOIETIC PROGENITORS IN THE SPLEEN AND FEMUR OF LETHALLY IRRADIATED MICE BY RLV-INFECTED W/W^V STEM CELLS
(9 days post-transplantation)

marrow cells injected	progenitors per spleen ($\times 10^3$) ± SD			progenitors per femur ($\times 10^3$) ± SD		
	CFU-E	GM-CFU	BFU-E	CFU-E	GM-CFU	BFU-E
10^7 W/W^V	9.6± 1.1	0.28±0.04	0.81±0.11	7.5±1.3	2.6±0.2	0.26±0.04
10^7 W/W^V RLV*	308.0±34	8.2 ±0.6	2.5 ±0.2	12.1±1.4	2.6±0.3	0.27±0.04
$\frac{W/W^V \text{ RLV}}{W/W^V}$	28	30	7.4	1.6	1.1	1.1

Lethally irradiated (9.0 Gy) BD_2-W/+ or W^V/+ littermates were used as recipients.
*BD_2 W/W^V mice, 21 days after RLV infection.
Results represent mean ± SD of three independent experiments.

Table 7.5

GENERATION OF HAEMOPOIETIC PROGENITORS IN THE SPLEEN OF LETHALLY IRRADIATED MICE BY RLV-INFECTED BD_2 +/+ CFC-S
(day 9 post-transplantation)

spleen cell donor	number of cells injected	colonies per spleen** ± SD	cells per colony ($\times 10^6$) ± SD	progenitors per colony ($\times 10^2$) ± SD		
				CFU-E	GM-CFU	BFU-E
BD_2 +/+	2.5×10^6	40 ± 6	1.3 ± 0.24	142 ± 22	1.2 ± 0.2	0.17 ± 0.06
BD_2 +/+ RLV*	2.5×10^6	49 ± 6	3.5 ± 0.5	620 ± 96	2.9 ± 0.4	0.44 ± 0.15

Lethally irradiated (9.0 Gy) BD_2-W/+ or W^V/+ littermates served as recipients.
* 3 weeks post RLV infection.
**Determined in mice which had received 5×10^5 spleen cells of the same suspension.
The number of cells per colony was calculated following the subtraction of spleen cell numbers obtained in lethally irradiated, untransplanted controls.
Data represent a mean of 2 individual experiments.

In order to further evaluate whether the enhancement of W/W^V stem cell differentiation in the spleen by RLV infection indeed represents a mechanism related to the expression of viral pathogenicity, the effect of RLV infection on progeny production in the spleen by +/+ stem cells, was investigated. These findings are shown in Table 7.5. Although less clear-cut by gross morphological inspection, a significant ($p < 0.01$) increase in the number of cells (3-fold) as well as progenitors per spleen colony (2-4 fold), formed by RLV-infected +/+ CFU-S, was observed.

The generation of haemopoietic progenitors in the femur of lethally irradiated mice transplanted with RLV-infected stem cells, is shown in Table 7.6. Again, no difference in progeny production due to RLV infection, could be found. Thus, RLV infection seems to enhance stem cell differentiation in the splenic environment only. This is true for primarily infected mice as well (3.1.2).

Table 7.6

GENERATION OF HAEMOPOIETIC PROGENITORS IN THE FEMUR OF LETHALLY IRRADIATED MICE BY RLV-INFECTED BD_2 +/+ STEM CELLS
(8 days post-transplantation)

marrow cell donor	number of cells injected	CFU-S injected ± SD**	progenitors per femur ($\times 10^3$) ± SD		
			CFU-E	GM-CFU	BFU-E
BD_2 +/+	3×10^5	76 ± 8	2.8 ± 0.4	1.1 ± 0.1	0.019±0.006
BD_2 +/+ RLV*	3×10^5	46 ± 5	1.9 ± 0.05	0.6 ± 0.05	0.014±0.001

Lethally irradiated (9.0 Gy) BD_2-W/+ or W^V/+ littermates were used as recipients.
* 3 weeks post-RLV-infection.
**Determined in mice which had received 5×10^4 marrow cells of the same suspension.

7.4 CONCLUSIONS

Several important observations related to the regulation of stem cell differentiation and self renewal, have been provided by the studies presented in this chapter, the findings of which are summarized in Table 7.7.

Table 7.7

THE EFFECT OF THE W MUTATION AND RLV-INFECTION ON STEM CELL DIFFERENTIATION AND SELF RENEWAL

	BD_2-W/W^V				BD_2-+/+			
	spleen		femur		spleen		femur	
property examined	W/W^V	W/W^V-RLV	W/W^V	W/W^V-RLV	+/+	+/+-RLV	+/+	+/+-RLV
differentiation	-	+	+	+	+	++	+	+
self-renewal	-	-	-	-	+	+	+	+

\+ normal
\- decreased
++ elevated

Assessment of progeny production by stem cells of W/W^V mutants under controlled experimental conditions, has provided conclusive evidence that the expression of defective stem cell differentiation due to the W mutation is determined by the environment in which these cells are situated. These data, supported by earlier observations of comparatively lower frequencies of megakaryocytes and erythroid precursors in the spleen of certain W/W^V strains (Chervenik and Boggs, 1969; Harrison and Russell, 1972; Gregory and Eaves, 1978), the relatively greater impairment in generation of erythroid cells in this organ after sublethal irradiation, hypoxia and red cell loss (Russell, 1963; Harrison and Russell, 1972), as well as that of haemopoietic progenitors in lethally irradiated hosts (Sutherland et al., 1970; Wagemaker and Visser, 1979), not only confirm the role of extrinsic influences in regulating stem cell differentiation, but further indicate that these influences differ at the major sites of haemopoiesis in the adult mouse.

Impairment of stem cell self renewal due to the W mutation, expressed in both femur and spleen, may explain the reduced frequency of stem cells in this mouse. Taken together with the data related to W/W^V stem cell differentiation, these observations strongly suggest that both stem cell properties are independently controlled. This is supported by the restoration of defective W/W^V stem cell differentiation and spleen colony growth by RLV infection, but not of self renewal.

Finally, by demonstrating that RLV infection restores the differentiation defect of W/W^V stem cells, while leaving their self replication defective, these observations argue against a homology between the SFFV proviral sequences and the normal sequences at the W-locus.

CHAPTER VIII

GENERAL DISCUSSION

The rapidity of induction, as well as the extreme reproducibility of the ensuing pathological manifestations, have made erythroproliferative disorders induced by murine retroviruses an attractive model in which to study the mechanisms involved in the disruption of normal regulation of haemopoiesis, resulting in fatal neoplastic disease.

For the virologist, delineation of the retroviral components which initiate the development of erythroid hyperplasia, has recently been successfully met. The development of highly sophisticated techniques for obtaining molecularly cloned fragments or deletion mutants of retroviral genomes (Linemeyer et al., 1981; 1982; Ruta and Kabat, 1980; Bestwick et al., 1973; Machida et al., 1984), has provided strong evidence for the role of the SFFV recombinant envelope gene, in this phenomenon. The molecular basis of the biological effects observed following viral infection, however, has not yet been established.

For the experimental haematologist, the search for a putative target cell, whose modified expression by SFFV infection may be primarily involved in the subsequent expression of viral pathogenicity, has been an exceedingly complex area of research, as well as dispute. One of the major limitations to the resolution of this issue and evaluation of the validity of its controversial concepts, has arisen from the continuous spread of virus among a variety of haemopoietic cells, thereby limiting a clear analysis of the effect of viral infection on the expression of individual cell types and their contribution to the ensuing pathological changes. Since viral spread among haemopoietic cells cannot be avoided following the infection of normal mice with the viral complex, it was with the aim of circumventing this limitation, that studies of the susceptibility of genetically anemic, stem-cell defective W/W^V mice to Rauscher infection, were initiated.

The development of typical Rauscher erythroblastosis in susceptible strains of W/W^V mice is characterized by a restoration of their stem cell spleen colony forming capacity (Chapter 3), thus providing direct evidence for the modification of stem cell expression by the virus. The major contribution of enhanced stem cell differentiation, resulting in macroscopic spleen colony formation to the subsequent expression of viral pathogenicity, is strongly supported by the highly reduced capacity of W/W^V stem cells to generate progeny in the spleen (McCulloch et al., 1964; Lewis et al., 1967; Bennett et al., 1968; Wagemaker and Visser, 1979; Chapter 7), together with the prominent expansion of haemopoiesis in this organ following RLV infection (Rauscher, 1962; Brommer, 1972; OKunewick, 1973; Seidel and Opitz, 1978; Hasthorpe, 1978; Chapter 3). The similar effect of RLV infection on splenic

differentiation of stem cells from normal mice (Chapter 7), lends further support to this hypothesis.

Based upon these observations, Rauscher disease may be proposed to result from a primary stem cell disorder. This is schematically described in Figure 8.1. Proviral expression within the stem cell genome results in enhanced differentiation in the spleen. In contrast to the hypothesis presented by Brommer (Brommer, 1972; Brommer and Bentvelzen, 1973), this event is not restricted exclusively to the erythroid compartment. Providing a major influx of all cell types to the progenitor cell pool, the concept of multiple targets within different lineages (OKunewick, 1977), can now be reduced to one common denominator. Its major expression within the erythroid compartment, thus mainly expressed as erythroblastosis, may be related to the specific environment in which enhanced stem cell differentiation is expressed (Chapter 7). Although poorly understood, strong evidence for a role of the splenic microenvironment in providing a preferentially inductive stimulus for stem cell differentiation into the erythroid compartment, has long been provided (Trentin et al., 1967; Wolf and Trentin, 1968). Possible alterations within the environment itself as a result of viral infection, such as the elevation of BFA levels, although not of primary relevance (Chapter 3), may be instrumental in the subsequent amplification of erythropoiesis (Walma and Wagemaker, 1979; Peschle et al., 1983; Chapter 6), as well. Elevated levels of erythropoietin, detected in Rauscher-infected mice (OKunewick and Erhard, 1974), are probably related to an effect of the virus, as yet unclear, on the formation of aberrant erythrocytes of increased osmotic fragility and highly reduced lifespan (de Both et al., 1978; 1980). Since increased numbers of megakaryocytes in the

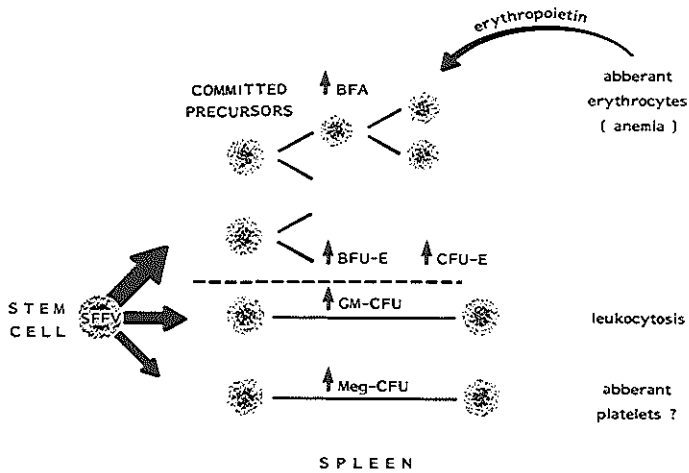


Figure 8.1:
Proposed scheme for the primary role of the stem cell in Rauscher erythroblastosis.

spleen following RLV infection have also been detected (Brommer, 1972), thrombocytopenia is probably a secondary phenomenon in Rauscher disease, as well.

Clonal assays in vitro have failed to provide an explanation to the mechanism(s) by which the W mutation or RLV infection exert their effects on stem cell expression. Identical ranges in sensitivity to known haemopoietic regulators have been observed for W/W^V haemopoietic cells. Although suggesting that a lesion in responsiveness to known regulators is not induced by the W mutation, the SAF responsiveness of W/W^V stem cells, found to be defective in self renewal, could not be evaluated. Furthermore, it is not clear at present whether all regulators which stimulate the initial stages of stem cell differentiation, have yet been identified. This possibility also applies to studies of the responsiveness of haemopoietic cells to various regulators following RLV infection. In relation to the W lesion, an additional possibility, difficult to determine in vitro, is that W/W^V stem cells may be more sensitive than normal stem cells to inhibitory influences. This is supported by the selective expression of the W/W^V stem cell differentiation defect in the spleen, which is, under normal conditions, a less favourable site for haemopoiesis than the femur. Whatever the lesion may be, these studies emphasize the presently limited contribution of in vitro culture systems to the fundamental understanding of the mechanisms involved in regulation of haemopoietic cell differentiation within the organism itself, and its disruption in disease.

The restriction of enhanced differentiation of both genetically anemic W/W^V as well as +/+ stem cells to the spleen following RLV infection (Chapter 7), demonstrates that expression of the SFFV component within haemopoietic stem cells is determined by the environment in which they are situated. Although in accordance with the tissue-tropism of Rauscher virus, this phenomenon is not easy to explain.

Endogenous xenotropic viruses have been found to be present in various mammalian tissues (Levy, 1978; reviewed by Pincus, 1980). Their conservation throughout evolution in the host genome, as well as the organ-specific differences in expression of viral proteins (Levy, 1978; Lerner, 1978), have led to various speculations regarding a possible role for these sequences in developmental processes.

Acquisition of the tissue-specific pathogenic properties of the erythroblastosis-inducing SFFV component is related to its formation by a recombination between the ecotropic MuLV helper and endogenous xenotropic viral sequences (reviewed by Troxler et al., 1980). In retrospect, a strong correlation between the restricted target organs of various such recombinant retroviruses and the tissue of their primary isolation, in which they probably evolved, is clearly apparent (Rauscher, 1962; Hartley et al., 1977; Ruscetti et al., 1981; Scolnick, 1982; Cloyd, 1983). Although the biological function of endogenous xenotropic viral genes has not yet been elucidated, the possibility that the acquired SFFV-related sequences are involved in organ-specific developmental processes during embryogenesis, is strongly implied by the major role of the liver and spleen in embryonic (Metcalf and Moore, 1970), as well as in RLV-induced (Rauscher, 1962; Brommer, 1972), haemopoiesis.

SUMMARY

Genetically anemic, stem cell-defective W/W^V mice on a DBA-2 or BD_2 F1 background have been found to be susceptible to infection with Rauscher Leukemia Virus (RLV). W/W^V mice exhibit the characteristic symptoms of splenomegaly, erythroblastosis, leukocytosis, more profound anemia, and thrombocytopenia. The development of Rauscher erythroblastosis in mice carrying the W mutation is characterized by a partial repair of their stem cell differentiation defect, resulting in the capacity of RLV-infected W/W^V spleen and marrow cell suspensions to give rise to macroscopically visible colonies on the spleen of a lethally irradiated host. These colonies are normal by both gross morphological as well as histological criteria (Chapter 3). The pluripotent nature of the macroscopic spleen colony forming cells is established by transplantation studies, demonstrating that equivalent numbers of RLV-infected W/W^V and +/+ CFU-S are equally effective in providing 30-day protection from lethal irradiation (Chapter 4).

The inability to detect macroscopic spleen colony formation by spleen and marrow cell suspensions of erythroblastosis-resistant W/W^V mice of C57BL genotype, supports an etiological link between the enhancement of stem cell differentiation and the development of Rauscher disease (Chapter 3). By using genetically resistant C57BL genotypes, the role of the SFFV component within the viral complex in repair of the macroscopic spleen colony forming capacity of W/W^V stem cells is strongly implied.

The seeding efficiency of the stem cells in the spleen was normal: neither RLV infection nor the W-mutation appeared to exert an influence. On the basis of this observation the self-replication capacity of the W/W^V CFU-S was determined and found to be ten-fold lower than normal. This finding explained the low number of CFU-S in RLV-infected W/W^V mice.

Restoration of the W/W^V stem cell differentiation defect by RLV infection is expressed in the capacity of these cells to give rise to erythrocytes of normal number and size distribution in lethally irradiated W/W^V recipients.

Clonal assays *in vitro*, performed in serum-free cultures specifically designed for the detection of haemopoietic stem cells and progenitor cells in vitro, have failed to demonstrate an alteration in the sensitivity of these cells to known haemopoietic regulators, as a result of either Rauscher infection or the W mutation (Chapter 6).

In vivo studies related to the effect of RLV infection on W/W^V stem cell behaviour (Chapter 7) have shown that the virus enhances the differentiation of haemopoietic stem cells, while leaving their self renewal defective and unaltered in normal mice. These findings provide evidence that both stem cell properties are under independent genetic control.

Restoration of W/W^V stem cell differentiation, while leaving their self replication defective, further suggests that the W gene is not homologous to the SFFV component.

Enhancement of stem cell differentiation following RLV infection, was observed for both W/W^V and +/+ stem cells (Chapter 7). These observations

lend further support for the primary role of the stem cell in Rauscher disease. The effect of RLV infection on stem cell differentiation is observed in the splenic environment only.

In view of the studies presented in this thesis, a model for the primary role of the stem cell in Rauscher erythroblastosis, is presented. The mainly erythroid expression of this disease is related to the environment in which enhanced stem cell differentiation is expressed. Whereas leukocytosis and erythroblastosis are primary manifestations of enhanced stem cell differentiation, anemia and thrombocytopenia are secondary characteristics, possibly related to the presence of virus within the mature end cells.

SAMENVATTING

Infektie van muizen met een erfelijke anemie, veroorzaakt door mutaties in het W-locus (W/W^V muizen), met Rauscher Leukemie Virus leidt, evenals bij normale muizen, tot erythroleukemie, gekenmerkt door splenomegalie, erythroblastose, leukocytose, meer ernstige anemie en thrombocytopenie. Het stamceldefect van W/W^V muizen komt onder meer tot uiting door een gebrekkige en daardoor macroscopisch niet zichtbare miltkolonievorming in lethaal bestraalde ontvangers. Infektie met RLV herstelt dit stamceldefect gedeeltelijk: injectie van RLV-geinfecteerde beenmerg- of miltsuspensies van W/W^V muizen leidde tot macroscopisch zichtbare miltkolonies. Het aantal miltkolonies was sterk gereduceerd ten opzichte van het aantal geproduceerd door beenmerg of milt van normale muizen. De samenstelling van de miltkolonies was evenwel histologisch normaal.

Het pluripotente karakter van de cellen die de miltkolonies vormen (CFU-S) werd onderzocht door het aantal granulocyte/macrofaag en erythroide voorlopercellen per miltkolonie te bepalen en door na te gaan hoeveel RLV-W/W^V CFU-S nodig zijn voor bescherming van een letaal bestraalde muis tegen dood door beenmergaplasie. Zowel het aantal voorlopercellen per miltkolonie, als het vermogen tot bescherming van een letaal bestraalde muis bleek equivalent te zijn aan normale CFU-S.

De seeding efficiency van de stamcellen in de milt was normaal: RLV-infectie noch de W-mutatie waren hierop van invloed. Met dit gegeven kon ook het vermogen tot zelfreplicatie van de W/W^V CFU-S worden bepaald. Dit bleek ongeveer tien maal lager te zijn dan normaal. Hiermee werd een verklaring gevonden voor het lage aantal CFU-S bij RLV-geinfecteerde W/W^V muizen.

Ongeinfecteerde W/W^V muizen hebben een macrocytaire anemie. Wanneer W/W^V muizen worden bestraald en vervolgens worden getransplanteerd met een suspensie van RLV-geinfecteerde W/W^V miltcellen, bereikt het aantal erythrocyten het niveau van de controles, terwijl ook de erythrocyten normaal van grootte werden. Tevens bleek in een lang overlevende muis herstel te zijn opgetreden van de weefsel-miltcellen. Hieruit blijkt, dat het herstel van de stamcellen door infectie met RLV niet beperkt blijft tot macroscopische miltkolonievorming.

Aangezien het effect van infektie met RLV op de stamcellen niet optrad bij W/W^V muizen van de C57BL-stam die genetisch resistent is tegen de SFFV-component van het RLV-complex, kan worden geconcludeerd dat, ten eerste, er mogelijk een ethiologisch verband bestaat tussen het effect van RLV op de stamcellen en de ontwikkeling van de erythroblastose en, ten tweede, het defektieve SFFV hoogstwaarschijnlijk verantwoordelijk is voor het gedeeltelijke herstel van de stamcellen van de W/W^V muis.

Ten behoeve van dit onderzoek werden serumvrije kweken uitgewerkt voor de detektie van hemopoietische stamcellen en voorlopercellen in vitro om na te gaan of infektie met RLV tot een gewijzigde gevoeligheid voor hemopoëse regulators leidt. In geen van deze assays kon evenwel een verschui-

ving in de gevoeligheid voor deze regulators worden aangetoond als gevolg van de W-mutatie of infektie met RLV.

Verdere bepalingen toonden aan dat RLV-infektie leidt tot een toenemende produktie van voorlopercellen bij een gelijkblijvend vermogen tot zelfreplicatie. De waarneming doet veronderstellen dat deze eigenschappen van de stamcel onder afzonderlijke genetische regulatie staan. Het herstel van het vermogen tot differentiatie van de W/W^V stamcellen bij defekt blijvende zelfreplikatie kan voorts worden opgevat als een aanwijzing dat het SFFV niet homoloog is aan het gen waarin de W- mutatie is opgetreden.

De toegenomen produktie van voorlopercellen tengevolge van infektie met RLV kon niet alleen worden aangetoond bij de W/W^V stamcellen, maar ook bij de +/+ controles. Deze observaties geven steun aan de primaire rol van de stamcel bij de ontwikkeling van Rauscher erythroblastose. Het effect van RLV-infektie op stamceldifferentiatie kon alleen in de milt worden waargenomen.

Op basis van de experimentele gegevens beschreven in dit proefschrift wordt een model gepresenteerd uitgaande van de primaire rol van de stamcel bij de ontwikkeling van Rauscher erythroblastose. De in hoofdzaak erythroide expressie van de ziekte is een direkte consequentie van de microomgeving van de milt waarin de vergrote produktie voornamelijk erythroide van voorlopercellen tot expressie komt. Terwijl de leukocytose en de erythroblastose hiermee rechtstreeks kunnen worden begrepen, moeten de anemie en de thrombocytopenie worden opgevat als secundaire verschijnselen, mogelijk te wijten aan de aanwezigheid van het virus in de rijpe eindcellen.

ACKNOWLEDGEMENTS

The work described in this thesis could not have been possible without the support and help of the following colleagues at the REPGO-Institutes, to whom I am greatly indebted:

- Prof.Dr. D.W. van Bekkum, for his inspiration and useful criticism during the investigation and the preparation of the manuscript, and for giving me the opportunity to complete the investigations at this institute.
- Dr. G. Wagemaker, for introducing me into the field of experimental haematology and for his constant encouragement, guidance and stimulating conversations throughout the investigations, all of which were greatly instrumental to the realization of this thesis.
- Dr. P. Bentvelzen, for supplying the Rauscher and Friend virus stocks and for his criticism in the preparations of the manuscripts.
- Dr. L. Dorssers for his participation in discussing experimental data.
- Mrs. E. Blauw, Mrs. J. Bart-Baumeister and Miss T.P. Visser for their enthusiastic and excellent technical assistance.
- Mr. H. Burger for constantly supplying purified effector molecules for these studies.
- Mr. J.C. Westhoff and Mr. J.I.M. van Hooft for expert breeding and supplying of the W/W^{v} mice.
- Mr. W. Slingerland and Mrs. J.W. Soekarman for performing peripheral blood analyses.
- Mr. E. Offerman and the histology laboratory staff at the IVEC for preparing histological sections.
- Mrs. M. van der Sman, Mrs. D. de Keizer, Mr. H. van Westbroek, Mr. J.Ph. de Kler and Mr. A. Glaudemans for their patient work in preparation of this and other manuscripts.
- Mrs. S. Knaan for her endless and indispensable friendship when times were hard.

The work described in this thesis is part of a study on the regulation of haemopoiesis, supported by a program grant of the Netherlands Foundation for Medical Research (FUNGO), which is subsidized by the Netherlands Organization for the Advancement of Pure Research (ZWO) and by a subsidy of the Netherlands Cancer Foundation "Koningin Wilhelmina Fonds".

ABBREVIATIONS

BFA	burst forming activity
BFU-E	burst forming units - erythroid
CEA	colony enhancing activity
CFC-S	colony forming cells - spleen
CFU-E	colony forming units - erythroid
CFU-GEMM	colony forming units - granulocyte-erythroid-macrophage-megakaryocyte
CFU-S	colony forming units - spleen
env	envelope
EP	erythropoietin
FCS	fetal calf serum
FLV-P	Friend leukaemia virus - polycythemic strain
GM-CFU	granulocyte-macrophage colony forming units
GM-CSF	granulocyte-macrophage colony stimulating factor
HBSS	Hanks' balanced salt solution
HSC	haemopoietic stem cell
M-CSF	macrophage colony stimulating factor
MCV	mean corpuscular volume
MuLV	murine leukaemia virus
RLV	Rauscher leukaemia virus (complex)
SAF	stem cell activating factor
SFFU	spleen focus-forming units
SFFV	spleen focus-forming virus

REFERENCES

deAberle, S. (1927). A study of the hereditary anemia of mice. Am. J. Anat. 40, 219.

Abramson, S., Miller, R.G. and Phillips, R.A. (1977). The identification in adult bone marrow of pluripotent and restricted stem cells of the myeloid and lymphoid systems. J. Exp. Med. 145, 1567.

Axelrad, A.A., McLeod, D.L., Shreeve, M.M., Heath, D.S. (1974). Properties of cells that produce erythrocytic colonies in vitro. In: Proceedings of the second International Workshop on Haemopoiesis in Culture. W.A. Robinson, ed. (DHEW Publ. NIH 74205), 226.

Baines, P. and Visser, J.W.M. (1983). Analysis and separation of murine bone marrow cells by H3342 fluorescence-activated cell sorting. Exp. Hemat. 11, 709.

Barnes, D. and Sato, G. (1980). Methods for growth of cultured cells in serum free medium. Anal. Biochem. 102, 255.

Becker, A.J., McCulloch, E.A. and Till, J.E. (1963). Cytological demonstration of the clonal nature of spleen colonies derived from transplanted mouse marrow cells. Nature 197, 452.

Bennett, M., Cudkowitz, G., Foster, R.S. and Metcalf, D. (1968). Haemopoietic progenitor cells of W anemic mice studied in vivo and in vitro. J. Cell. Physiol. 71, 211.

Bentvelzen, P., Aarsen, A.M. and Brinkhof, J. (1972). Defectivity of Rauscher murine erythroblastosis virus. Nature 239, 122.

Bernstein, S.E. and Russel, E.S. (1959). Implantation of normal blood forming tissue in genetically anemic mice, without X-irradiation of host. Proc.Soc.Exp.Biol.Med. 101, 769.

Bestwick, R., Ruta, M., Kiessling, A., Faust, C., Linemeyer, D., Scolnick, E.M. and Kabat, D. (1983). Genetic structure of Rauscher spleen focus-forming virus. J. Virol. 45, 1217.

Blackett, N.M. (1974). Statistical accuracy to be expected from cell colony assays; with special reference to the spleen colony assay. Cell Tissue Kinet. 7, 407.

Bleiberg, I., Liron, M. and Feldman, M. (1965). Reversion by erythropoietin of the suppression of erythroid clones caused by transfusion-induced polycythemia. Transplantation 3, 706.

Boiron, M., Levy, J.P., Lasneret, J. and Oppenheim, S. (1965). Pathogenesis of Rauscher leukemia. J. Nat. Canc. Inst. 35, 865.

Bol, S. and Williams, N. (1980). The maturation state of three types of granulocyte/macrophage progenitor cells from mouse bone marrow. J. Cell. Physiol. 102, 233.

Bol, S., Visser, J.W.M. and Van den Engh, G. (1977). Physical characterization of haemopoietic progenitor cells by equilibrium density centrifugation. In: Cell Separation Methods. Bloemendal, H., ed., Elsevier/North-Holland Biomedical Press, Amsterdam, 39.

Bol, S., Visser, J.W.M. and Van den Engh, G. (1979). The physical separation of three subpopulations of granulocyte/macrophage progenitor cells from mouse bone marrow. Exp. Hemat. 7, 541.

Bonsdorff, E. and Jalavisto, E. (1948). A humoral mechanism in chronic erythrocytosis. Act. Physiolog. Scand. 16, 150.

de Both, N.J., Vermey, M. and Van Griensven, L.J.L.D. (1978). The effect of Rauscher leukemia virus on the haemopoietic system of BALB/c mice. Cell proliferation and cell loss. Exp. Haemat. 6, 515.
de Both, N.J., Vermey, M., van 't Hull, E., Klootwijk-van Dijke, E., van Griensven, L.J.L.D., Mol, J.N.M. and Stoof, T.J. (1978a). A new erythroid cell line induced by Rauscher murine leukemia virus. Nature 272, 626.
de Both, N.J., Klootwijk-van Dijke, E., Vermey, M. and de Bruijn, W.C. (1980). Influence of Rauscher leukemia virus infection on the lifespan of erythrocytes in BALB/c mice. In: Experimental Hematology Today 1980. Baum, S.J., Ledney, G.D., Van Bekkum, D.W., eds., Karger, S., Basel., p. 157.
Bradley, T.R. and Metcalf, D. (1966). The growth of mouse bone marrow cells in vitro. Aust. J. Exp. Biol. Med. Sci. 44, 287.
Bradley, T.R. and Sumner, M.A. (1968). Stimulation of mouse bone marrow colony growth in vitro by conditioned medium. Austr. J. Exp. Biol. Med. Sci. 46, 607.
Bradley, T.R., Stanley, E.R. and Sumner, M.A. (1971). Factors from mouse tissues stimulating colony growth of mouse bone marrow cells in vitro. Aust. J. Exp. Biol. Med. Sci. 49, 595.
Brommer, E.J.P. (1972). The role of the stem cell in Rauscher murine leukemia. Thesis, Erasmus University, Rotterdam.
Brommer, E.J.P. and Bentvelzen, P. (1973). Interactions between differentiation and leukemogenesis in Rauscher Murine Leukemia. In: Unifying Concepts of Leukemia. Bibl. Haemat. 39, Dutcher, R.M. and Chieco-Bianchi, L., Eds., S. Karger, Basel, p. 929.
Brommer, E.J.P. and Bentvelzen, P. (1974). The haemopoietic stem cell in Rauscher virus-induced erythroblastosis of BALB/c mice. Eur. J. Cancer 10, 827.
Brouwer, A., Burger, H. and Wagemaker, G. (1980). Purification of properties of colony stimulating factors isolated from pregnant mouse uteri. REP-TNO Annual Report, p. 39.
Brozovic, B., Sljivic, V.S. and Warr, G.W. (1975). Haematological changes and iron metabolism in rats after administration of Corynebacterium parvum. Br. J. Exp. Path. 56, 183.
Burgess, A.W. and Metcalf, D. (1980). The nature and action of granulocyte-macrophage colony stimulating factors. Blood 56, 947.
Burgess, A.W., Camakaris, J. and Metcalf, D. (1977). Purification and properties of colony-stimulating factor from mouse lung-conditioned medium. J. Biol. Chem. 252, 1998.
Burgess, A.W., Metcalf, D., Nicola, N.A. and Russell, S.H.M. (1978). Purification and characterization of cell specific colony stimulating factors. In: Hematopoietic Cell Differentiation. Golde, D.W., Cline, M.J., Metcalf, D., Fox, C.F., eds. New York Academic Press, 399.
Byron, J.W. (1972). Comparison of the action of 3H-thymidine and hydroxyurea on testerosterone treated haemopoietic cells. Blood 40, 178.
Byron, J.W. (1972a). Evidence for a β-adrenergic receptor initiating DNA synthesis in haemopoietic stem cells. Exp. Cell. Res. 71, 228.
Byrne, P., Heit, W. and Kubanek, B. (1977). The in vitro differentiation of density subpopulations of colony forming cells under the influence of different types of colony stimulating factor. Cell Tissue Kinet. 10, 341.
Carnot, P. and Deflandre, C. (1906). Sur l'activité hémopoietique du serum au cours de la regeneration du sang. Compt. Rend. Acad. Sci. 143, 384.

Cerny, I. (1974). Stimulation of bone marrow haemopoietic stem cells by a factor from activated T cells. Nature 249, 63.
Chesebro, B., Portis, J.L., Wehrly, K. and Nishio, J. (1983). Effect of murine host genotype on MCF virus expression, latency and leukemia cell type of leukemias induced by Friend Murine Leukemia helper virus. Virology 128, 221.
Chervenik, P.A. and Boggs, D.R. (1969). Decreased neutrophils and megakaryocytes in anemic mice of genotype W/W^V. J. Cell. Physiol. 73, 25.
Cloyd, M.W. (1983). Characterization of target cells for MCF viruses in AKR mice. Cell 32, 217.
Cooper, R.A., Arner, E.C., Wiley, J.S. and Shattil, J.S. (1975). Modification of red cell membrane structure by cholesterol-rich lipid dispersions. A model for the primary spur cell defect. J. Clin. Invest. 55, 115.
Crowle, P.K. (1982). Bone marrow origin of mucosal mast cells. Fed. Proc. 41, 581.
Curry, J.L. and Trentin, J.J. (1967). Hemopoietic spleen colony studies. I. Growth and differentiation. Dev. Biol. 15, 395.
Dorssers, L., Burger, H. and Wagemaker, G. (1984). Identity of murine stem cell activating factor (SAF) and interleukin-3 (IL3) and common specificity for pluripotent haemopoietic stem cells. Exp. Hematol. 12, A65.3, 357.
Duesburg, P.H. (1979). Transforming genes of retroviruses. In: Cold Spring Harbor Symp. Quant. Biol. 44, 33.
Dunn, Th.B. and Green, A.W. (1966). Morphology of BALB/c mice inoculated with Rauscher leukemia virus. J. Nat. Canc. Inst. 36, 987.
Dunn, Th.B., Malmgren, P.C., Carney, P.C. and Green, A.W. (1966). PTU and transfusion modification of the effects of Rauscher virus in BALB/C mice. J. Nat. Canc. Inst. 36, 1003.
Ebbe, S. and Phalen, E. (1978). Regulation of megakaryocytes in W/W^V mice. J. Cell. Physiol. 96, 73.
Ebbe, S., Phalen, E. and Stohlman, F. (1973). Abnormalities of Megakaryocytes in W/W^V mice. Blood 42, 857.
Fagg, B., Ostertag, W., Klein, B. and le Bousse, C. (1983). Myeloproliferative Sarcoma Virus: Its effects on erythropoiesis in adult DBA/2 mice. J. Cell. Physiol. 116, 16.
Ford, C.E., Hamerton, J.L., Barnes, D.W. and Loutit, J.F. (1956). Cytological identification of radiation chimeras. Nature 177, 452.
Foster, R., Metcalf, D., Robinson, W.A. and Bradley, T.R. (1968). Bone marrow colony stimulating activity in human serum. Brit. J. Haematol. 15, 147.
Friend, C. (1957). Cell free transmission in adult Swiss mice of a disease having the character of leukemia. J. Exp. Med. 105, 307.
Fung, M.C., Hapel, A.J., Ymer, S., Cohen, D.R., Johnson, R.M., Campbell, H.D., Young, I.G. (1984). Molecular cloning of cDNA for murine interleukin-3. Nature 307, 233.
Gallicchio, V.S. and Murphy, M.J. (1981). The role of erythropoietin in erythroid colony formation from Rauscher leukemia virus-infected mice. Exp. Hemat. 9, 938.
Garland, J.M. and Crampton, S. (1983). A preliminary report: Preparations containing Interleukin-3 (IL-3) promote proliferation of multipotential stem cells (CFU-S) in the mouse. Exp. Hemat. 11, 757.

Gregory, C.J. (1976). Erythropoietin sensitivity as a differentiation marker in the hemopoietic system: Studies of three erythropoietic colony responses in culture. J. Cell. Physiol. 89, 289.
Gregory, C.J. and Eaves, A.C. (1978). Three stages of erythropoietic progenitor cell differentiation distinguished by a number of physical and biological properties. Blood 51, 527.
Gregory, C.J., Tepperman, A.D., McCulloch, E.A. and Till, J.E. (1974). Erythropoietic progenitors capable of colony formation in culture: Response of normal and genetically anemic W/W^V mice to manipolations of the erythron. J. Cell. Physiol. 84, 1.
Gross, M. (1980). The control of protein synthesis by hemin in rabbit reticulocytes. Mol. Cell. Biochem. 31, 25.
Guilbert, L.J. and Iscove, N.N. (1976). Partial replacement of serum by selenite, transferrin, albumin and lecithin in haemopoietic cell cultures. Nature 263, 594.
Hagemeijer, A., Smit, E.M.E., Govers, F. and de Both, N.J. (1982). Trisomy 15 and other nonrandom chromosome changes in Rauscher murine leukemia virus-induced leukemia cell lines. J. Nat. Cancer Inst. 69, 945.
Hankins, D.W. (1983). Increased erythropoietin sensitivity after in vitro transformation of hematopoietic precursors by RNA tumor viruses. J. Nat. Canc. Inst. 70, 725.
Hara, H. and Ogawa, M. (1978). Murine haemopoietic colonies in culture containing normoblasts, macrophages and megakaryocytes. Am. J. Hematol. 4, 23.
Harrison, D.E. (1972). Lifesparing ability (in lethally irradiated mice) of W/W^V mouse marrow with no macroscopic colonies. Radiat. Res. 52, 553.
Harrison, D.E. and Russell, E.S. (1972). The response of W/W^V and S1/S1d anemic mice to haemopoietic stimuli. Br. J. Haematol. 22, 155.
Harrison, D.E., Malathi, V.G. and Silber, R. (1975). Elevated erythrocyte nucleoside deaminase levels in genetically anemic W/W^V and S1/S1d mice. Blood Cells 1, 614.
Hartley, J.W., Wolford, N.K., Old, L.J. and Rowe, R.P. (1977). A new class of leukemia virus associated with development of spontaneous lymphoma. Proc. Nat. Acad. Sci. 74, 789.
Hasthorpe, S. (1978). Kinetics of erythroid and myeloid CFU following Rauscher virus infection. In: In vitro aspects of erythropoiesis. Murphy, M.J. Jr., ed., Springer-Verlag, New York, p. 172.
Hasthorpe, S. and Bol, S. (1979). Erythropoietin responses and physical characterization of erythroid progenitor cells in Rauscher virus infected BALB/c mice. J. Cell. Physiol. 100, 77.
Horoszewicz, J.S., Leong, S.S., Carter, W.A. (1975). Friend leukemia. Rapid development of erythropoietin-independent haematopoietic precursors. J. Nat. Cancer Inst. 54, 265.
Humphries, R.K., Jacky, P.B., Dill, F.J. and Eaves, A.C. (1979). CFU-S in individual erythroid colonies derived in vitro from adult mouse marrow. Nature 279, 718.
Humphries, R.K., Eaves, A.C., Eaves, C.J. (1981). Self-renewal of hemopoietic stem cells during mixed colony formation in vitro. Proc. Nat. Acad. Sci. 78, 3629.
Ibrahim, N.G., Lutton, J.D., Levere, R.D. (1982). The role of haem biosynthetic and degradative enzymes in erythroid colony development: the effect of haemin. Br. J. Haematol. 50, 17.

Ichikawa, Y., Pluznik, D.H. and Sachs, L. (1966). In vitro control of the development of macrophage and granulocyte colonies. Proc. Nat. Acad. Sci. 56, 488.
Iscove, N.N. (1978). Erythropoietin-independent stimulation of early erythropoiesis in adult marrow cultures by conditioned media from lectin-stimulated mouse spleen cells. In: Hemopoietic Cell Differentiation. Cline, M.J., Golde, D.W., Metcalf, D. and Fox, C.F., eds., Academic Press, New York, p. 37.
Iscove, N.N. (1979). Committed erythroid precursor populations in genetically anemic W/W^V and S1/S1d mice. Proc. First Int. Symp. Aplastic Anemia. Hibino, S., Takaku, F. and Shahidi, N.T., eds., Univ. Tokyo Press, p. 31.
Iscove, N.N., Till, J.E. and McCulloch, E.A. (1972). Studies on the significance and properties of human CFU-C. In: In vitro culture of hemopoietic cells. Van Bekkum, D.W. and Dicke, K.A., eds., Proceedings of Workshop/Symposium, Radiobiological Institute TNO, Rijswijk, The Netherlands, p. 270.
Iscove, N.N., Sieber, F. and Winterhalter, K.H. (1974). Human urinary erythroid colony stimulating activity: assay in cultures of mouse and human bone marrow, and similarity to erythropoietin on Sephadex and Con A Sepharose. In: Hemopoiesis in Culture. W.A. Robinson, ed., DHEW Publ. No. (NIH), 74.205, 44.
Iscove, N.N., Guilbert, L.J. and Weyman, C. (1980). Complete replacement of serum in primary cultures of erythropoietin-dependent red cell precursors (CFU-E) by albumin, transferrin, iron, unsaturated fatty acid, lecithin and cholesterol. Exp. Cell. Res. 126, 121.
Iturriza, R.G. and Seidel, H.J. (1974). Stem cell growth and production of colony-stimulating factor in Rauscher virus-infected CBA/J mice. J. Nat. Cancer Inst. 53, 487.
Johnson, G.R. and Metcalf, D. (1977). Pure and mixed erythroid colony formation in vitro stimulated by spleen conditioned medium with no detectable erythropoietin. Proc. Nat. Acad. Sci. 74, 3879.
Johnson, G.R. (1980). Colony formation in agar by adult bone marrow multipotential haemopoietic cells. J. Cell. Physiol. 103, 371.
Jolicouer, P. and Baltimore, D. (1976). Effect of FV-1 gene product on proviral DNA formation and integration in cells infected with murine leukemia viruses. J. Virol. 16, 1593.
Keighley, G.H., Lowy, P., Russell, E.S. and Thompson, M.W. 81966). Analysis of erythroid homeostatic mechanisms in normal and genetically anemic mice. Br. J. Haematol. 12, 461.
Kitamura, Y., Shimada, M., Hatanaka, K. and Miyano, Y. (1977). Development of mast cells from grafted bone marrow cells in irradiated mice. Nature 268, 442.
Kitamura, Y., Go, S. and Hatanaka, K. (1978). Decrease of mast cells in W/W^V mice and their increase by bone marrow transplantation. Blood 52, 447.
Kitamura, Y., Yokoyama, M., Matsuda, H., Ohno, T. and Mori, K.J. (1981). Spleen colony-forming cell as common precursor for tissue mast cells and granulocytes. Nature 291, 159.
Klein, B., Le Bousse, C., Fagg, B., Smajda-Joffe, F., Vehmeyer, K., Mori, K.J., Jasmin, C. and Ostertag, W. (1981). Effects of myeloproliferative sarcoma virus on the pluripotential stem cell and granulocyte precursor cell populations of DBA/2 mice. J. Nat. Canc. Inst. 66, 935.

Korn, A.P., Henkelman, R.M., Ottensmeyer, F.P. and Till, J.E. (1973). Investigations of a stochastic model of haemopoiesis. Exp. Hemat. 1, 362.

Koury, M.J. and Pragnell, I. (1982). Retroviruses induce granulocyte-macrophage colony stimulating activity in fibroblasts. Nature 299, 638.

Kreja, L. and Seidel, H.J. (1981). Role of BPA and erythropoietin for BFU-E growth from Friend virus-infected mice. Stem Cells 1, 367.

Kretchmar, A.L. and Conover, W.R. (1970). A difference between spleen derived and marrow derived colony forming units in ability to protect lethally irradiated mice. Blood 36, 772.

Kubota, K., Motoyoshi, K., Kajikaya, S., Suda, T., Saito, M. and Miura, Y. (1983). Morphological examonations of murine granulocyte/macrophage colonies grown in serum free cultures. Exp. Hematol. 11, 364.

Lahiri, S.K. and Van Putten, L.M. (1969). Distribution and multiplication of colony forming units from bone marrow and spleen after injection in irradiated mice. Cell Tissue Kinet. 2, 21.

Lahiri, S.K., Keizer, H.J. and Van Putten, L.M. (1970). The efficiency of the assay for haemopoietic colony forming cells. Cell Tissue Kinet. 5, 365.

Lerner, R.A. (1978). Recombinant origins of leukemogenic murine viruses. Am. J. Pathol. 95, 10.

Levy, J.A. (1978). Xenotropic type C viruses. In: Current Topics in Microbiology and Immunology 79, Basle, W.A., Ed., Springer- Verlag, Berlin, Heidelberg, New York, p. 111.

Lewis, J.P. and Trobaugh, F.E. (1964). Haematopoietic stem cells. Nature 204, 589.

Liao, S. and Axelrad, A.A. (1975). Erythropoietin-independent erythroid colony formation in vitro by haemopoietic cells of mice infected with Friend virus. Int. J. Cancer 15, 467.

Lilly, F. and Pincus, T. (1973). Genetic control of murine viral leukemogenesis. Adv. Canc. Res. 17, 231.

Linemeyer, D.L., Ruscetti, S.K., Scolnick, E.M., Evans, L.H. and Duesberg, P.H. (1981). Biological activity of the spleen focus forming virus is encoded by a molecularly cloned subgenomic fragment of spleen focus-forming virus DNA. Proc. Natl. Acad. Sci. 78, 1401.

Linemeyer, D.L., Menke, J.G., Ruscetti, R.K., Evans, L.H. and Scolnick, E.M. (1982). Envelope gene sequences which encode the gp52 protein of spleen focus-forming virus are required for the induction of erythroid cell proliferation. J. Virol. 43, 223.

Little, C.C. and Cloudman, A.M. (1937). The occurrence of a dominant spotting mutation in the house mouse. Proc. Natl. Acad. Sci. U.S.A. 23, 535.

Lord, B.I. and Hendry, J.H. (1973). Observations on the settling and recoverability of transplanted haemopoietic colony forming units in mouse spleen. Blood 41, 409.

Löwenberg, B. (1975). Fetal liver transplantation. Academic Thesis, Rotterdam.

Löwenberg, B. and Dicke, K.A. (1976). Studies on the in vitro proliferation of pluripotent haemopoietic stem cells. In: Leukemia and aplastic anemia. Proc. International Conference on Leukemia and Aplastic Anemia, Naples.

Löwenberg, B. and Dicke, K.A. (1977). Induction of proliferation of haemopoietic stem cells in culture. Exp. Hemat. 5, 319.

Lushbaugh, C.C. and Russell, E.S. (1963). Complete change of circulating erythrocytes in W/W^{V} genetically anemic mice receiving +/+ normal blood forming tissue. Blood 22, 500A.

MacDonald, M.E., Reynolds, F.H., Van de Ven, W.J., Stephenson, J.R., Mak, T.W. and Bernstein, A. (1980a). Anemia and polycythemia-inducing isolates of Friend spleen focus-forming virus: Biological and molecular evidence for two distinct viral genomes. J. Exp. Med. 151, 1477.

MacDonald, M.E., Johnson, G.R. and Bernstein, A. (1980b). Different pseudotypes of Friend spleen focus-forming virus induce polycythemia and erythropoietin-independent colony formation in serum free medium. Virology 110, 231.

Machida, C.A., Bestwick, R.H. and Kabat, D. (1984). Reduced leukemogenicity caused by mutations in the membrane glycoprotein gene of Rauscher spleen focus-forming virus. J. Virol. 49, 394.

Magli, M.C., Iscove, N.N. and Ordatchenko, N. (1982). Transient nature of early hemopoietic spleen colonies. Nature 295, 527.

Mak, T.W., Axelrad, A.A. and Bernstein, A. (1979). The FV-2 locus controls expression of Friend spleen focus-forming virus specific sequences in normal and infected mice. Proc. Nat. Acad. Sci. U.S.A. 76, 5809.

Marchal, G. and Milon, G. (1981). Decreased erythropoiesis: The origin of BCG induced anemia in mice. Br. J. Hematol. 48, 551.

McCulloch, E.A. and Till, J.E. (1963). Repression of colony-forming ability of C57BL hemopoietic cells transplanted into non-isologous hosts. J. Cell. Comp. Physiol. 61, 301.

McCulloch, E.A., Siminovitch, L. and Till, J.E. (1964). Spleen colony formation in anemic mice of genotype W/W^{V}. Science 144, 844.

Metcalf, D. (1981). Haemopoietic colony stimulating factors. In: Tissue growth factors. Baserga, R., Ed., Springer-Verlag, Berlin, Heidelberg, New York, p. 343.

Metcalf, D. and Burgess, A.W. (1981). Clonal analysis of progenitor cell committment to granulocyte or macrophage production. J. Cell. Physiol. 111, 275.

Metcalf, D. and Foster, R. (1967). Behaviour on transfer of serum stimulated bone marrow colonies. Proc.Soc.Exp.Biol.Med. 126, 758.

Metcalf, D. and Johnson, G.R. (1978). Production by spleen and lymph node cells of conditioned medium with erythroid and other hemopoietic colony stimulating activity. J. Cell. Physiol. 96, 31.

Metcalf, D. and Merchav, S. (1982). Effects of GM-CSF deprivation on precursors of granulocytes and macrophages. J. Cell. Physiol. 112, 411.

Metcalf, D. and Moore, M.A.S. (1971). Hemopoietic Cells. In: Frontiers of Biology Vol. 24, Neuberger, A. and Tatum, E.L., Eds., North- Holland Publishing Company, Amsterdam.

Metcalf, D. and Stanley, R. (1967). Quantitative studies on the stimulation of mouse bone marrow colony growth in vitro by normal human urine. Austr. J. Exp. Biol. Med. Sci. 47, 453.

Metcalf, D., Parker, J., Chester, H.M. and Kincade, P.W. (1974). Formation of eosinophilic granulocytic colonies by mouse bone marrow cells in vitro. J. Cell. Physiol. 84, 275.

Metcalf, D., MacDonald, M., Ordatchenko, N. and Sordat, B. (1975). Growth of mouse megakaryocyte colonies in vitro. Proc. Nat. Acad. Sci. U.S.A. 72, 1744.

Metcalf, D., Warner, N.L., Nossal, S.J.V., Miller, J.F.A.P., Shortman, K. and Rabellino, E. (1975a). Growth of B lymphocyte colonies in vitro from mouse lymphoid organs. Nature 255, 630.

Mirand, E.A. (1967). Virus induced polycythemia in mice: erythropoiesis without erythropoietin. Science 156, 832.
Monette, F.C., Holden, S.A. (1982). Hemin enhances the in vitro growth of primitive erythroid progenitor cells. Blood 60, 527.
Nakahata, T., Spicer, S.S., Cantey, J.R. and Ogawa, M. (1982). Clonal assay of mouse mast cell colonies in methylcellulose cultures. Blood 60, 352.
Nakeff, A., Dicke, K.A. and Van Noord, M.J. (1975). Megakaryocytes in cultures of mouse bone marrow. Ser. Haematol. 8, 1.
Nicola, N.A., Metcalf, D., Melchner, H. von and Burgess, A.W. (1981). Isolation of murine fetal haemopoietic progenitor cells and selective fractionation of various erythroid precursors. Blood 58, 346.
Niece, R.L., MacFarland, E.C. and Russell, E.S. (1963). Erythroid haemostasis in normal and genetically anemic mice: Reaction to induced polycythemia. Science 142, 1468.
Nowell, P.C., Hirsch, B.E., Fox, D.H. and Wilson, D.B. (1970). Evidence for the existence of multipotential lymphohaemopoietic stem cells in the adult rat. J. Cell Physiol. 75, 151.
OKunewick, J.P. (1977). The role of committed and uncommitted haemopoietic stem cells as targets for Rauscher and Friend leukemia virus. Biomedicine 26, 152.
OKunewick, J.P. and Chervenich, P.A. (1977). Sequential response of pluripotent and committed haematopoietic colony forming cells to Rauscher leukemia virus. Proc. Soc. Exp. Biol. Med. 155, 382.
OKunewick, J.P. and Erhard, P. (1974). Accelerated clearance of exogenously administered erythropoietin by mice with Rauscher viral leukemia. Cancer Res. 34, 917.
OKunewick, J.P. and Phillips, E.L. (1973). Change in marrow and spleen CFU compartments following leukemia virus infection: comparison of Friend and Rauscher Virus. Blood 42, 885.
OKunewick, J.P., Fulton, D. and Markoe, A.M. (1972). Radioprotective effect of Rauscher Leukemia Virus in the SJL/J mouse. Radiat. Res. 49, 521.
Opitz, U., Seidel, H.J. and Risch, I.S. (1977). Erythroid stem cells in Rauscher virus infected mice. Blut 35, 35.
Ostertag, W. and Pragnell, I.B. (1981). Differentiation and viral involvement in differentiation of transformed mouse and rat erythroid cells. Current Topics in Microbiology and Immunology 94/95, 143.
Ostertag, W., Pragnell, I.B., Fusco, A., Hughes, D., Freshney, M., Klein, B., Jasmin, C., Bilello, J., Warnecke, G. and Vehmeyer, K. (1982). Studies on the biology and genetics of murine erythroleukemia viruses. In: Expression of Differentiated Functions in Cancer Cells. Revoltella, R.F., ed., Raven Press, New York, p. 56.
Paran, M. and Sachs, L. (1968). The continued requirement for inducer for the development of macrophage and granulocyte colonies. J. Cell. Physiol. 72, 247.
Peschle, C., Rossi, G.B., Migliacchio, G., Migliacchio, A.R. and Mastroberardino, G. (1983). Friend virus erythroleukemias: A tentative model for the erythropoietic preleukemic stage. In: Recent Advances in Experimental Hematology. Tonella et al., Eds., Academic Press, New York.
Pincus, T. (1980). The endogenous murine type C viruses. In: Molecular Biology of RNA Tumor Viruses. Stephenson, J.R., Ed., Academic Press, New York, p. 77.

Pluznik, D.H. and Sachs, L. (1964). Quantitation of a murine leukemia virus with a spleen colony assay. J. Nat. Cancer Inst. 33, 535.
Pluznik, D.H. and Sachs, L. (1966). The induction of colonies of normal "mast" cells by substance in conditioned medium. Exp. Cell Res. 43, 553.
Pluznik, D.H., Sachs, L. and Resnitsky, P. (1966). The mechanism of leukemogenesis by the Rauscher leukemia virus. Nat. Canc. Inst. Monogr. 22, 3.
Porter, P.N., Meints, R.H., Mesner, K. (1979). Enhancement of erythroid colony growth in culture by hemin. Exp. Hematol. 7, 11.
Rauscher, F.J. (1962). Virus-induced disease of mice characterized by erythrocytopoiesis and lymphoid leukemia. J. Nat. Canc. Inst. 29, 515.
Reddy, P., Dunn, C.Y. and Aaronson, S.A. (1980). Different lymphoid cell targets for transformation by replication-competent Moloney and Rauscher leukemia viruses. Cell 19, 663.
Robinson, W.A., Bradley, T.R. and Metcalf, D. (1967). Stimulation by normal and leukemic mouse sera of colony formation in vitro by mouse bone marrow cells. J. Cell. Physiol. 69, 83.
Rossi, G.B. and Peschle, C. (1980). Enhanced proliferation and migration of BFU-E and erythropoietin independence of CFU-E expression in FLV-infected mice: Comparative studies on anemic and polycythemic strain. In: In vivo and in vitro erythropoiesis. The Friend System. Rossi, G.B., ed., Amsterdam, Elsevier/North Holland, p. 139.
Rous, P. (1910). A transmissible avian neoplasm (sarcoma of the common fowl). J. Exp. Med. 12, 696.
Rozenszaijn, L.A., Shoham, D. and Kalechman, I. (1975). Clonal proliferation of PHA-stimulated human lymphocytes in soft agar culture. Immunology 29, 1041.
Ruscetti, S., Davis, L., Feild, J. and Oliff, A. (1981). Friend murine leukemia virus-induced leukemia is associated with the formation of mink cell focus-inducing xenotropic viral envelope genes. J. Exp. Med. 154, 907.
Russell, E.S. and Bernstein, S.E. (1966). Blood and blood formation. In: The Biology of the Laboratory Mouse. Green, E.L., Ed., McGraw Hill, New York, 351.
Russell, E.S., Smith, L.J. and Lawson, F.A. (1956). Implantation of normal blood forming tissue in radiated genetically anemic hosts. Science 124, 1956.
Ruta, M. and Kabat, D. (1980). Plasma membrane glycoproteins encoded by cloned Rauscher and Friend spleen focus-forming viruses. J. Virol. 35, 844.
Schofield, R., Lord, B.I., Kyffin, S. and Gilbert, C.W. (1980). Self-maintenance capacity of CFU-S. J. Cell. Physiol. 103, 355.
Schrader, J.W. and Clark Lewis, T. (1982). A T cell-derived factor stimulating multipotential hemopoietic stem cells: molecular weight and distribution from T cell growth factor and T cell-derived granulocyte-macrophage colony stimulating factor. J. Immunol. 124, 30.
Scolnick, E.M. (1982). Hyperplastic and neoplastic erythroproliferative diseases induced by oncogenic murine retroviruses. Biochim. Biophys. Acta 651, 273.
Seidel, H.J. (1972). Pancytopenia in CBA mice after Rauscher virus infection. J. Nat. Cancer Inst. 48, 959.
Seidel, H.J. (1973). Target cell characterization for Rauscher leukemia virus in vivo. In: Unifying Concepts of Leukemia. Bibl. Hemat. 39, Dutcher, R.M. and Chieco-Bianchi, L., eds., 935.

Seidel, H.J. and Opitz, U. (1978). Comparative studies on haemopoietic stem cell pools in mice after Friend (FV-P) or Rauscher Virus infection. In: In Vitro Aspects of Erythropoiesis. Murphy Jr., M.J., Ed., p. 143.
Siminovitch, L., McCulloch, E.A. and Till, J.E. (1963). The distribution of colony forming cells among spleen colonies. J. Cell. Comp. Physiol. 62, 327.
Stanley, E.R., Metcalf, D., Maritz, J.S. and Yeo, G.F. (1972). Standardized bioassay for bone marrow colony stimulating factor in human urine. Levels in normal man. J. Lab. Clin. Med. 79, 657.
Stanley, E.R. and Heard, P.M. (1977). Factors regulating macrophage production and growth: Purification and some properties of colony stimulating factor purified from medium conditioned by mouse L cells. J. Biol. Chem. 252, 4305.
Steeves, R.A. (1975). Spleen focus-forming virus in Friend and Rauscher leukemia virus preparations. J. Nat. Canc. Inst. 54, 289.
Stephenson, J.R., Axelrad, A., McLeod, D.L. and Shreeve, M.M. (1971). Induction of colonies of hemoglobin-synthesizing cells by erythropoietin in vitro. Proc. Nat. Acad. Sci. 68, 1542.
Sutherland, D.J.A., Till, J.E. and McCulloch, E.A. (1970). A kinetic study of the genetic control of hemopoietic progenitor cells assayed in culture and in vivo. J. Cell Physiol. 75, 267.
Testa, N.G., Lord, B.I. and Shore, N.A. (1972). The in vivo seeding of haemopoietic colony forming cells in irradiated mice. Blood 40, 654.
Till, J.E. and McCulloch, E.A. (1961). A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. Radiat. Res. 14, 213.
Till, J.E., McCulloch, E.A. and Siminovitch, L. (1964). A stochastic model of stem cell proliferation, based on the growth of spleen colony-forming cells. Proc. Nat. Acad. Sci. 51, 29.
Todaro, G.J. (1980). Interspecies transmission of mammalian retroviruses. In: Molecular Biology of RNA Tumor Viruses. Stephenson, J.R., Ed., Acad. Press, New York, p. 47.
Toth, F.D., Vaczi, L. and Balogh, M. (1973). Inheritance of susceptibility and resistance to Rauscher leukemia virus. Acta Microbiol. Acad. Sci. Hung. 20, 183.
Trentin, J.J. and Fahlberg, W.J. (1963). An experimental model for studies of immunological competence in irradiated mice repopulated with "clones" of spleen cells. In: Conceptual advances in Immunology and Oncology, 16th Annual Symp. Texas, Harper Row, New York, p. 66.
Trentin, J.J., Curry, J.C., Wolf, N. and Cheng, V. (1967). Factors controlling stem cell differentiation and proliferation: The haemopoietic inductive microenvironment (HIM). In: The proliferation and spread of neoplastic cells. M.D. Anderson Hospital, 21st Am. Symp. on Fund. Canc. Res., Baltimore, Williams and Wilkins, p. 713.
Troxler, D.H., Boyars, J.K., Parks, W.R. and Scolnick, E.M. (1977). Friend strain of spleen focus-forming virus: a recombinant between mouse type C ecotropic viral sequences and sequences related to xenotropic virus. J. Virol. 22, 361.
Troxler, D.H., Ruscetti, S.K. and Scolnick, E.M. (1980). The molecular biology of Friend virus. Biochim. Biophys. Acta 605, 305.
Toujas, L., Dazord, L., Le Garrec, Y. and Sabolovic, D. (1972). Modifications du nombre d'unites formatrices de colonies spleniques par des bacteries induisant l'immunostimulatin non specifique. Experientia 28, 1223.

Van Bekkum, D.W. (1977). The appearance of the multipotential haemopoietic stem cell. In: Experimental Haematology Today. Baum, S.J. and Ledney, G.D., Eds., Springer Verlag, New York, Heidelberg, Berlin, p. 3.
Van Bekkum, D.W. and Dicke, K.A. (1972). Haemopoietic stem cells in bone marrow of W/W^{V} mice. REP-TNO Annual Report, p. 168.
Van Bekkum, D.W., Van den Engh, G., Wagemaker, G., Bol, S.J.L. and Visser, J.W.M. (1979). Structural identity of the pluripotential haemopoietic stem cell. Blood Cells 5, 143.
Van den Engh, G. and Bol, S. (1975). The presence of a CSF enhancing activity in the serum of endotoxin-treated mice. Cell Tissue Kinet. 8, 579.
Vassort, F., Winterholer, M., Frindel, E. and Tubiana, M. (1973). Kinetic parameters of bone marrow stem cells using in vivo suicide by tritiated thymidine or by hydroxyurea. Blood 41, 789.
Vogel, H., Niewisch, H. and Matioli, G. (1968). The self renewal probability of haemopoietic stem cells. J. Cell. Physiol. 72, 221.
Vos, O., Davids, J.A.G, Weyzen, W.W.H. and Van Bekkum, D.W. (1956). Evidence for the cellular hypothesis in radiation protection by mouse bone marrow cells. Acta Physiol. Pharmacol. Neerl. 4, 482.
Wagemaker, G. (1978). Cellular and soluble factors influencing the differentiation of primitive erythroid progenitor cells (BFU-E) in vitro. In: In vitro aspects of erythropoiesis. Murphy Jr., M.J., Ed., Springer-Verlag, New York, p. 44.
Wagemaker, G. (1980). Early erythropoietin-independent stage of in vitro erythropoiesis: relevance to stem cell differentiation. In: Experimental Hematology Today. Baum, S.J., Ledney, G.D. and Van Bekkum, D.W., Eds., S. Karger, Basel, Munchen, Paris, London, New York, Sydney, p. 47.
Wagemaker, G. (1980a). Erythropoietin independent regulation of early erythropoiesis. In: In vivo and in vitro erythropoiesis: the Friend system. Rossi, G.B., Ed., Elsevier/North Holland Biomedical Press, Amsterdam, p. 87.
Wagemaker, G. (1981). Hemopoietic factors required for differentiation of multipotential cells in vitro. In: Hemoglobins in development and differentiation. Stamatoyannopoulos, G. and Nienhuis, A.W., Eds., New York, Alan R. Liss, p. 85.
Wagemaker, G. and Burger, H. (1982). Specificity of granulocyte/macrophage colony stimulating factors. REP-TNO Annual Report, p. 197.
Wagemaker, G. and Peters, M.F. (1978). Effects of human leukocyte conditioned medium on mouse haemopoietic progenitor cells. Cell Tissue Kinet. 11, 45.
Wagemaker, G. and Visser, T.P. (1980). Erythropoietin-independent regeneration of erythroid progenitor cells following multiple injections of hydroxyurea. Cell Tissue Kinet. 13, 505.
Wagemaker, G., Merchav, S. and Burger, H. (1982). Multiplication of pluripotent haemopoietic stem cells induced by purified stem cell activating factor (SAF) in serum free suspension cultures. Exp. Hemat. 10 (suppl. 11)., 162.
Walma, E.P. and Wagemaker, G. (1979). Early regulatory disturbance of erythropoiesis in Rauscher disease. Exp. Hemat. 7 (suppl. 6), 77.
Weitz-Hamburger, A., Fredrickson, T.N., LoBue, J., Hardy, W.D., Ferdinand, P. and Gordon, D.S. (1973). Inhibition of erythroleukemia in mice by induction of haemolytic anemia prior to infection with Rauscher leukemia virus. Cancer Res. 33, 104.

Wiktor-Jedrzejczak, W., Sharkis, A., Ahmed, A., Sensenbrenner, L. and Sell, K.W. (1979). Engraftment of bone marrow transplants in W anemic mice measured by electronic determination of the red blood cell size profile. Exp. Hemat. 7, 416.

Williams, N. and Eger, R.R. (1978). Purification and characterization of clonable murine granulocyte/macrophage precursor cell populations. In: Hematopoietic Cell Differentiation. Cline, M., Golde, D.W., Metcalf, D., Fox, C.D., Eds., Academic Press, New York, p. 385.

Wolf, N.S. and Trentin, J.J. (1968). Haemopoietic colony studies. V. Effect of hemopoietic organ stroma on differentiation of pluripotent stem cells. J. Exp. Med. 127, 205.

Worton, R.G., McCulloch, E.A. and Till, J.E. (1969). Physical separation of hemopoietic stem cells from cells forming colonies in culture. J. Cell. Physiol. 74, 171.

Wu, A.M., Till, J.E., Siminovitch, L. and McCulloch, E.A. (1968). Cytological evidence for a relationship between normal hematopoietic colony forming cells and cells of the lymphoid system. J. Exp. Med. 127, 455.

Yokota, T., Lee, F., Rennick, D., Hall, L., Arai, N., Mossmann, T., Nabel, G., Cantor, H., Arai, K. (1984). Isolation and characterization of a mouse cDNA clone that expresses mast-cell growth-factor activity in monkey cells. Proc. Natl. Acad. Sci. USA 81: 1070.

CURRICULUM VITAE

Shoshana Merchav was born in Jerusalem, Israel, in 1951. She completed her biology studies at the Tel Aviv University (B.Sc.) in 1972. In 1974, she pursued her studies in the field of Immunology, at the Technion Medical Faculty in Haifa. Her M.Sc. research project, performed under the supervision of Prof. H. Gershon, involved *in vitro* studies related to cellular immune functions in aging mice. She received her M.Sc. degree in 1977. Thereafter, she was involved in several projects related to the effect of various mycoplasma strains on lymphocyte stimulation in rodents, performed under the supervision of Dr. Y. Naot.
In 1980 she joined the Radiobiological Institute TNO in Rijswijk, where she was introduced to the field of experimental haematology by Dr. G. Wagemaker. During her stay at this Institute, the investigations described in this thesis were performed.
In 1983, she entered the Department of Haematology at the Technion Medical Faculty in Haifa.